FIRST-STAGE AND SINGLE-STAGE CONTINUOUSLY STIRRED TANK ANAEROBIC DIGESTION OF SYNTHETIC COMPLEX WASTEWATER AND PIGGERY WASTEWATER (WITH EMPHASIS ON THERMOPHILIC TEMPERATURE)

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DECLARATION

I declare that this thesis is my own account of my research work undertaken which has not been previously submitted for a degree at any tertiary educational institution

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ABSTRACT

Single-stage continuously stirred tank reactor (CSTR) is commonly used in the anaerobic treatment of animal manure slurry, municipal sewage sludge and concentrated wastewaters containing a high proportion of biodegradable particulate organic materials at relatively long hydraulic retention times (HRTs) of 12 to 24 days. It is also commonly used as a holding tank to equilise the big variations in wastewater flow or pollution strength as well as for pre-acidification of wastewater. Its simplicity, ease of operation, low capital and maintenance costs are appealing features that made it a natural choice of reactor configuration for the pilot-scale thermophilic first-stage acidogenic digester of a two-stage thermophilic-ambient anaerobic digestion system which is based at Roseworthy Campus of University of Adelaide, South Australia and operated by South Australian Research and Development Institute (SARDI).

As the first-stage acidogenic reactor plays a crucial role in the solubilisation of particulate organic matter in complex substrates to soluble organics and acidification to volatile fatty acids (VFAs) for enhancing pathogens destruction in wastewater treatment, the initial aim of this research study was to optimise the first-stage anaerobic CSTR to effectively convert particulate-containing complex organic wastewater to intermediate fermentation products for feed to the second-stage methane reactor. Pig feed pellets was used as the model substrate to prepare the complex synthetic wastewater to investigate the effects of temperature (37, 47 and 55^{o}C) and pH (6, 7 and 8) on organics solubilisation and acidification in two sets of batch vial experiments while the effects of HRT (4- to 1-day) on organics conversion efficiency of the semi-continuous first-stage anaerobic CSTRs were investigated in two sets of experiments conducted at mesophilic (37^{o}C) and thermophilic (55^{o}C) conditions. Findings from the batch vial experiments with low organic strength (4 g/L TCOD) wastewater found mesophlic temperature at 37^{o}C and pH 7-8 were optimum conditions for solubilisation (hydrolysis) and acidification than at thermophilic temperatures of 47^{o}C and 55^{o}C. Results from the semi-continuous CSTR anaerobic reactors confirmed that the mesophilic (37^{o}C) reactor hydrolysed

and acidified significantly more particulate and soluble organic matter respectively than thermophilic (55^{o}C) reactor, with 2-day HRT being the optimum for both the anaerobic acidogenic reactors. The lack of methane in the biogas which contained hydrogen and carbon dioxide confirmed that the methanogens present in the acidic reactor effluents were completely inhibited.

Following reports that the pilot-scale first-stage thermophilic (55^{o}C) acidogenic reactors treating raw piggery wastewater was experiencing substantial lost of volatile fatty acids to methane formation at relatively short HRTs of 4 and 7-day, the complex synthetic wastewater was replaced with real piggery wastewater from Roseworthy Campus's piggery holding sump to allow meaningful lab-scale reactor experiments to be conducted in order to provide realistic information for the pilot-scale acidogenic reactor. Semi-continuous first-stage anaerobic reactor experiments were carried out to examine the influence of piggery influent concentrations with and without pH reduction on the fermentation behaviour of the thermophilic acidogenic reactor at a shorter HRT of 2-day. The 2-day HRT was found to be optimum in previous acidogenic experiments with the synthetic complex wastewater.

The studies on the acidogenic treatment of piggery wastewater at 2-day HRT revealed that irrespective of the feed concentrations or organic loading rates, first-stage anaerobic treatment of the piggery wastewater without pH intervention could not completely inhibit the syntrophic acetogenic and methanogenic microorganisms because of the wastewater's inherently high levels of alkalinity and ammonia-nitrogen which buffered the system against VFA souring. Some losses of total VFAs were observed at the highest TCOD feed concentration of 13 g TCOD/L and OLR of 6.5 g/L/d. The vast differences in the physico-chemical and microbiological characteristics of the raw piggery wastewater and synthetic complex wastewater, particularly with respect to their initial ammonia-nitrogen, soluble COD, volatile fatty acids (VFA), buffering capacity and anaerobic microorganisms, were the key determining factors for the contradictory outcomes in organics conversion performance of the thermophilic and mesophilic first-stage CSTRs.

Although the study on pH reduction of the piggery wastewater to pH 5.5 found the approach was successful in suppressing the activities of syntrophic consortia of acetogenic and methanogenic microbial populations while stimulating the acidogenic

bacteria, the operational inconvenience from foaming-related spillages and the anticipated need to re-adjust the acidic effluent pH to neutral for feed to the second-stage reactor far out-weighed the small gains in the increased hydrolysis and acidification of the piggery influent organic matter.

The observations that around 30% of the organics still remained as insoluble particulate form in the treated effluent and more than 60% of the organic carbon compounds in the raw piggery wastewater was already in soluble and acidified forms coupled with its high buffering capacity which protects the anaerobic system against failure from VFA souring, it was decided that single-stage thermophilic anaerobic digestion at longer HRT of 10- and 15-day might be more cost-effective for enhancing the solubilisation of the particulate organics and organic carbon conversion to methane in the undiluted piggery wastewater. Semi-continuous thermophilic CSTR experiments at 55^{o}C were carried out to examine the extent of organic carbon conversion at 10- and 15-day HRT. Mesophilic CSTR experiment at 37^{o}C was also carried out to compare its organics conversion performance with the thermopilic reactor at 15-day HRT.

The results show that while increasing the HRT of the thermophilic anaerobic CSTRs from 2- to 10- and 15-day saw a gradual increase in specific methane yields, the methane yield at the longer HRT of 15-day was considered low (26% of total COD fed) based on the COD material balance of the digested effluents. Around 30% of the organic matter still remained as non-biodegradable particulate organics while propionate (19%) and unidentified non-VFA soluble organic matter (17%) formed the two largest groups of unconverted soluble organics in the digested piggery effluent. The build-up of propionate at higher HRT of 10- and 15-day which correlated positively with increased free ammonia concentration implied that the syntrophic propionate-oxidising bacteria and hydrogenotrophic microorganisms were under increased stress. At 15-day HRT, although anaerobic thermophilic digestion at 55^{o}C had significantly higher specific methane yield than mesophilic digestion at 37^{o}C, the chemical quality of thermophilic digested effluent was poor with regards to its higher levels of free ammonia, propionate, total VFA and soluble COD compared to the mesophilic effluent. However, thermophilic digestion is universally recognised for its higher pathogens destruction efficency than mesophilic digestion.

Five sets of thermophilic (55^{o}C) batch vial experiments were conducted to investigate the single effect of pH reduction, chemical (zeolite, humic acid) and biological (piggery biomass, municipal biomass) supplements as well as the combined effects of pH reduction and chemical or biological supplements in enhancing methane production from thermophilic piggery effluent. Reduction of the piggery effluent pH from 8.1 to 6.5 alone and zeolite treatment (10 to 20 g/L) with or without pH reduction of the piggery effluent to pH 6.5 were found to be effective strategies for enhancing methane production yet not elevating the effluent COD level compared to its initial level.

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LIST OF ABBREVIATIONS

APHA	American Public Health Association
atm	atmosphere
CSTR	Continuously Stirred Tank Reactor
CO_2	Carbon dioxide
d	day
FISH	Fluorescence *In Situ* Hybridisition
GC	Gas Chromatography
g	gram
HRT	Hydraulic Retention Time
hr	hour
H_2	Hydrogen
IBS	Integrated BioSystem
kg	kilogram
kJ	kiloJoule
L	Litre
mL	millilitre
µL	microlitre
mM	millimolar
mg	milligram
M	Molar
N	Normality
ng	nanogram
CH_4	Methane
OLR	Organic Loading Rate
PCR	Polymerase Chain Reaction
rRNA	ribosomal RiboNucleic Acid
rDNA	ribosomal DeoxyRibonucleic Acid
stp	standard temperature and pressure
ΔG^o	standard Gibbs free energy
SRB	Sulphate Reducing Bacteria

SRT	Solid Retention Time
SCOD	Soluble Chemical Oxygen Demand
SARDI	South Australian Research and Development Institute
TPAD	Temperature-Phased Anaerobic Digestion
TVFAs	Total Volatile Fatty Acids
TSS	Total Volatile Solids
TS	Total Solids
T-RFLP	Terminal-Restriction Fragment Length Polymorphism
TCOD	Total Chemical Oxygen Demand
VSS	Volatile Suspende Solids
VS	Volatile Solids

CHAPTER 1
INTRODUCTION

1.1. BACKGROUND

With growing public concern of waste pollution impacting on health and environmental quality, most authorities have responded by developing and implementing environmental policies and regulations that require waste generators to adopt best practicable waste management technologies to reduce their environmental footprints. One such technology is the engineered anaerobic digestion (AD) system that treats organic waste and wastewater for resource recovery and reuse. The AD system relies on a consortium of naturally-occurring mixed anaerobic microorganisms in the wastes to decompose organic matter to methane and carbon dioxide in an oxygen-free methanogenic environment. The process occurs naturally in many anoxic environments such as waterlogged soils, swamps, sediments, animal and human digestive guts (Ward *et al.*, 2008). Recognition of its natural ability to produce renewable biofuels (methane, hydrogen, alcohols) as alternative replacements for the unsustainable fossil fuels of petroleum hydrocarbon and coal (Ghosh, 1997) as well as its conservation of the fertiliser value in stabilised wastes and effluents in conjunction with pathogens destruction at thermophilic conditions (Bendixen, 1994) have spurred research and development into different types of contained anaerobic digestion systems to capture these beneficial features of AD for sustainable use and socially responsible waste management. To date, engineered anaerobic digestion has been in used for the treatment of low- to high-strength organic waste effluents from a variety of industries and agro-industrial sources such as food, beverage, paper pulp mill, palm oil mill, agricultural, petrochemical and animal waste effluents; domestic sludge and more recently the organic fraction of municipal solid wastes (Angelidaki *et al*., 2003; Demirel and Yenigun, 2002; Chynoweth *et al*., 1998).

The anaerobic biological conversion of complex organic matter to methane and carbon dioxide is a highly complex process. It involves the simultaneous action of a number of microbial populations linked by their individual substrate and product

specificities (Harper and Pohland, 1986). In the conventional single-stage anaerobic digester, all these microorganisms co-exist in a delicate relationship and participate in four successive biological processes of hydrolysis, acidogenesis, acetogenesis, methanogenesis or sulfinogenesis (de Lemos Chernicharo, 2007). Complex polymers (carbohydrates, proteins and lipids) are first converted into soluble monomers (sugars, amino aicds and fatty acids) by extracellular enzymes excreted by the hydrolytic bacteria during hydrolysis. These monomers are transformed into simple compounds such as volatile fatty acids (VFA), alcohols, hydrogen, carbon dioxide and ammonia by the acidogenic fermentative bacteria. These intermediate products excluding ammonia in turn are degraded by the synthropic consortium of acetogenic and methanogenic microorganisms to methane and carbon dioxide in a methanogenic environment (Harper and Pohland, 1986, Pavlostathis and Giraldo-Gomez, 1991) or hydrogen sulphide and carbon dioxide in a sufinogenic environment (de Lemos Chernicharo, 2007). Stable digester operation in the methanogenic environment requires the the acid-forming and methane-forming bacterial groups to be in balance. Because of their differences in growth kinetics, nutritional requirements and sensitivity to environmental conditions, imbalance between the two phases of acid fermentation and methane production commonly results in the accumulation of volatile fatty acids and other malodourous products which inhibit the methane-producing microorganisms in the conventional single-stage anaerobic digester.

The development of two-phase or two-stage anaerobic digestion system which consists of a first-stage acidogenic reactor and a second-stage methanogenic reactor was a technological strategy to overcome the process deficiency of the single-stage anaerobic digester (Pohland and Ghosh, 1971). Through manipulation of the reactors' operating conditions, optimum conditions could be created to favour the fast-growing acid-forming fermentative bacteria and the slow-growing syntrophic VFA-consuming acetogenic bacteria and methane-forming *archaea* to dominate in the first- and second-stage reactors respectively. Numerous studies have since demonstrated the efficiency of the two-stage anaerobic system (at mesophilic or thermophilic temperatueres for both serial reactors) in enhancing process stability particularly at high organic loading rates and increasing volatile solids destruction over single-stage system (Demirer and Chen, 2005; Pavan *et al.,* 2000; Banks and Wang, 1999; Hanaki *et al*., 1987, Fongastitkul *et al*., 1994; Cseh *et al*., 1984). In all

cases, anaerobic digestion at thermophilic conditions was found to facilitate higher organic loading rates, greater volatile solids removal and greater pathogens destruction than at mesophilic conditions although its chemical quality was poor compared to mesophilic digested effluent.

The observations of other potential applications of acid-phase anaerobic digestion, such as the generation of volatile fatty acids for use as carbon and energy sources in the enhanced biological nutrient removal system to facilitate microbial removal of nitrogen and phosphorus in the wastewater (Elefsiniotis *et al*., 2004; Maharaj and Elefsiniotis, 2001; Banerjee *et al*., 1998; Elefsiniotis and Oldham, 1993), production of hydrogen biogas as an alternative sustainable biofuel (Jan *et al.*, 2007; Lay *et al.*, 2005; Wu and Lin, 2004; van Ginkel *et al*., 2001, Okamoto *et al*., 2000), enhanced pathogens destruction to minimise environmental health risk (Kunte *et al*., 2004, Sung and Santha, 2001; Mitsdörffer *et al.*, 1990), and enhanced solubilisation of particulate organic solids (Eastman and Ferguson, 1981, Elefsiniotis *et al*., 1996) have led to numerous reseach efforts being targeted on the first-stage acidogenic reactor to fully exploit its potentials for these applications. One spring-off of the two-phase anaerobic system is the temperature-phased anaerobic digestion process (TPAD) developed by Dague and co-workers in the mid-nineties which consists of thermophilic (55^{o}C) first-stage acidogenic digester and mesophilic (35^{o}C) second-stage methanogenic digester to exploit the benefits of each digester for the treatment of wastewater sludges (Han *et al.*, 1997; Han and Dague, 1995; Kaiser and Dague, 1994). The TPAD system has since being extended to the treatment of livestock manures to enhance solubilisation of particulate organics and conversion of soluble organics to volatile fatty acids as well as enhanced pathogens destruction and improved final effluent quality (Harikishan and Sung, 2003; Sung and Santha, 2001).

It is this backdrop of environmental benefits of the two-stage anaerobic system that shaped the multi-disciplinary research project titled ‘Commercial scale integrated biosystem (IBS) for organic waste and wastewater treatment for the livestock and food processing industries’ led by South Australian Research and Development Institute (SARDI) into integratng a two-stage anaerobic treatment technology with resource recovery and reuse in the downstream aquaculture system. The aim of IBS is to develop an integrated farming system that prevents organic waste pollution

through an integrated waste treatment of anaerobic conversion of organic carbon and release of nutrients for reuse in the downstream aquaculture system, thereby enhancing the sustainability and profitability of the primary and secondary industries (www.ebcrc.com.au).

The pilot-scale IBS project is based in Roseworthy Campus at the University of Adelaide, South Australia. It utilises piggery wastewater generated at the Roseworthy Campus as the model livestock feedwater for treatment in a two-stage thermophilic-ambient anaerobic digestion system. Raw piggery wastewater collected from the holding sump is first treated in the thermophilic (55^{o}C) first-stage anaerobic acidogenic continuously-stirred tank reactors (CSTRs) at 7-day HRT to solubilise and acidify the organic matter. The acidified effluent is then treated in the ambient second-stage plug-flow methanogenic digestors (polybags) that operate at 23-day HRT. The nutrient-rich digested piggery effluent from the second-stage digesters is fed to the integrated aquaculture system that comprises the microalgae tank, zooplankton tank and the fish tank. Nutrients released in the digested effluent are utilised by the microalgae to grow and multiply. The microalgae serve as food source for the zooplankton which in turn grow and multiply and serve as food source for the cultured fish downstream. The valuable end products from the IBS are biofuel (methane), aquaculture fish production and treated waste effluent safe for reuse or disposal.

As one of the key objectives of the IBS project was to maximise solubilisation and acidification of the organic carbon components and release of nutrients (nitrogen and phosphorus) besides pathogens destruction in the thermophilic first-stage acidogenic digester, this thesis's research focus was thus aligned to it in order to gain a better insight into the factors influencing anaerobic treatment of complex organic wastewater in the anaerobic first-stage or acidognic CSTR. This research focus was later broadened to single-stage anaerobic digestion of raw piggery wastewater to enhance the overall organics conversion to methane and carbon dioxide.

In anaerobic digestion of complex organic wastes and wastewaters which typically contained a mixture of soluble and insoluble organic biopolymers (carbohydrates, proteins and lipids), hydrolysis of the insoluble particulate organic components is the rate-limiting process as it determines the amount of soluble substrate available for

the fermentative acidogens to convert to methanogenic substrates such as volatile fatty acids, hydrogen and carbon dioxide for degradation to methane and carbon dioxide by the terminal syntrophic consortia of acetogens and methanogens at any given retention time (Fox and Pohland, 1994; Pavlostathis and Giraldo-Gomez, 1991; McInerney, 1988; Eastman and Ferguson, 1981). Thus, it affects not just the acid-phase digestion but the overall process kinetics of a two-stage anaerobic digestion system. However, literature review of the acid-phase anaerobic digestion of complex organic wastewaters revealed that most of these studies had focused solely on the acidification of soluble organic components while hydrolysis of the undissolved organic polymeric materials was omitted (Yu and Fang, 2002, 2001; Dinopoulou *et al*., 1988a; Demirel and Yenigun, 2004; Ince, 1998). Consequently, very short HRT of 12 h was reported to be optimum for VFA production. In contrast, other researchers applied longer HRT that varied between 2 and 5 days on the first-stage acidogenic reactor of a two-stage anaerobic system treating animal wastewaters (Demirer and Chen, 2005; Ahn *et al*., 2004; Harikishan and Sung, 2003; Sung and Santha, 2001; Cseh *et al*., 1984) and sludge (Han *et al.*, 1997; Han and Dague, 1995; Kaiser and Dague, 1994) to enhance particulate organic matter and pathogens destruction.

Besides the rate-limiting particulate organic matter, livestock wastewaters and manure slurries also contain very high level of organic nitrogen that originates from the nitrogenous urea and proteinaceous materials. During anaerobic digestion, ammonium-nitrogen and dissolved free ammonia are released. The non-ionised free ammonia is widely known to be responsible for the inhibition of methanogenic process and limiting the organic loading rates of the anaerobic digesters treating animal wastes (Gallert *et al*., 1998; Hashimoto, 1983). Its concentration increases with increasing temperature, pH and total ammonia concentration. This explains why thermophilic anaerobic digestion of nitrogenous wastes and wastewaters tends to produce substantially higher effluent free ammonia concentration than mesophilic digestion. Ammonia toxicity is reported to occur at total ammonia (free ammonia plus ammonium) concentrations between 1.5 and 3.0 g/L-N at pH above 7.4 and above 3.0 g/L irrespective of pH values (McCarty, 1964) whilst free ammonia concentrations of 560 to 700 mg/L at pH above 7.4 were reported to inhibit methanogenesis under thermophilic conditions (Gallert and Winter, 1997; Angelidaki

and Ahring, 1994). Although high ammonia level is universally recognised for its inhibitory effect on methane production, it is unclear whether ammonia adversely affected the acetoclastic methanogens or hydrogenotrophic methanogens as contradictory reports have been presented (Hansen *et al*., 1998; Angelidaki and Ahring, 1994; Wiegant and Zeeman, 1986). Methanogenic cultures can be adapted to high ammonia levels with long HRT and high substrate loading (Angelidaki and Ahring, 1994; Braun *et al,* 1981).

Various means to reduce the ammonia inhibition on biogas production from animal wastes have been extensively investigated by numerous researchers and their findings were comprehensively reviewed by Yadvika *et al*. (2004) and Chen *et al.* (2008). These included reducing the reactor effluent pH, reactor temperature, diluting the digester feedwater, recirculating digested slurry to the reactor, process modification, gradual acclimatisation of the biomass with reduced organic loading rate, ammonium precipitation and recovery as struvite (an ammonium magnesium phosphate slow-release fertiliser), and additions of inorganic and organic additives such as zeolite, activated carbon, clay and iron to adsorb ammonium and ammonia.

1.2. OBJECTIVES OF THIS RESEARCH WORK

1. To examine whether the acidogenic anaerobic microorganisms cultivated at the transition temperature of 47^{o}C had the lowest organics conversion to VFAs compared to 37^{o}C (mesophilic optimum) and 55^{o}C (thermophilic optimum) as well as to establish the optimum pH for maximum organics solubilisation and acidification.

2. To establish the optimum hydraulic retention times (HRT) for maximum hydrolysis and acidification of the synthetic complex organic wastewater in the thermophilic (55^{o}C) and mesophilic (37^{o}C) semi-continuous acidogenic CSTRs.

3. To determine the extent of net hydrolysis and acidification of the raw piggery wastewater in the thermophilic (55^{o}C) and mesophilic (37^{o}C) first-stage acidogenic reactors and compare them with the synthetic complex wastewater at the same low HRT.

4. To investigate which operational factors (pH, piggery influent concentrations or organic loading rates) would affect the activity of the methanogenic microorganisms under thermophilic (55^{o}C) conditions in the first-stage acidogenic reactors at low HRT of 2-day.

5. To determine and compare the extent of net hydrolysis of particulate organic carbons and conversion of VFAs to methane at longer HRT of 10- and 15-day in relation to 2-day HRT under thermophilic (55^{o}C) conditions.

6. To determine the extent of net hydrolysis of particulate organic carbons and conversion of VFAs to methane under mesophilic (37^{o}C) conditions at 15-day HRT for comparison with its thermophilic counterpart.

7. To investigate the effects of pH, chemical and biological supplements in enhancing methane production from thermophilic digested piggery effluent under thermophilic (55^{o}C) conditions.

1.3. ORGANISATION OF THIS THESIS

Chapter 2 presents the literature review on topics relevant to this research work - microbiology and biochemistry of the anaerobic digestion, anaerobic reactor configurations and their applications, factors affecting the acid-phase and single-stage anaerobic digestion, and nucleic-acid based molecular methods for microbial analysis.

Chapter 3 covers the general materials and methods used in the experimental studies.

Chapter 4 covers the batch vial anaerobic acid-phase experiments on the synthetic complex organic wastewater.

Chapter 5 covers the semi-continuous acid-phase anaerobic CSTR experiments on the synthetic complex organic wastewater at varying short HRTs of 4-, 3-, 2- and 1-day.

Chapter 6 presents the semi-continuous anaerobic first-stage CSTR acidogenic experiments on the raw piggery wastewater without pH reduction and with pH reduction at short HRT of 2-day.

Chapter 7 presents the semi-continuous anaerobic single-stage CSTR experiments on the raw piggery wastewater at long HRTs of 10- and 15-day.

Chapter 8 presents the thermophilic batch vial anaerobic digestion experiments to mitigate ammonia inhibition and to enhance methane production from the thermophilic piggery effluent.

Chapter 9 presents the overall conclusions drawn from these experimental studies and recommendations for future research studies.

CHAPTER 2
LITERATURE REVIEW

2.1. INTRODUCTION

The following areas are reviewed in this chapter to provide background knowledge for the design of experiments and for interpreting experimental results in this thesis: 1) microbiology and biochemistry of anaerobic digestion process; 2) brief outline of the types of anaerobic reactor configurations, with particular emphasis on completely-stirred tank reactor (CSTR) as this reactor is used to treat piggery wastewater at South Australian Research Development Institute (SARDI)'s pilot trial in Adelaide; 3) factors affecting the acid-phase digestion of the anaerobic system; 4) factors affecting the single-stage anaerobic digestion, and 5) nucleic acid-based molecular methods for microbial analysis, with particular emphasis on fluorescence *in situ* hybridisation (FISH) method.

2.2. ANAEROBIC DIGESTION PROCESS

Anaerobic digestion is a biological conversion process undertaken in the absence of oxygen by several groups of microorganisms in the conversion of complex organic matter to final products such as methane, carbon dioxide, hydrogen sulphide and ammonia (de Lemos Chernicharo, 2007). These groups of microorganisms are characterised as prokaryotes and fall under the *Bacteria* and *Archaea* domains in Woese's universal phylogenetic or evolutionary tree of life as depicted in Figure 2.1. (Barton, 2005).

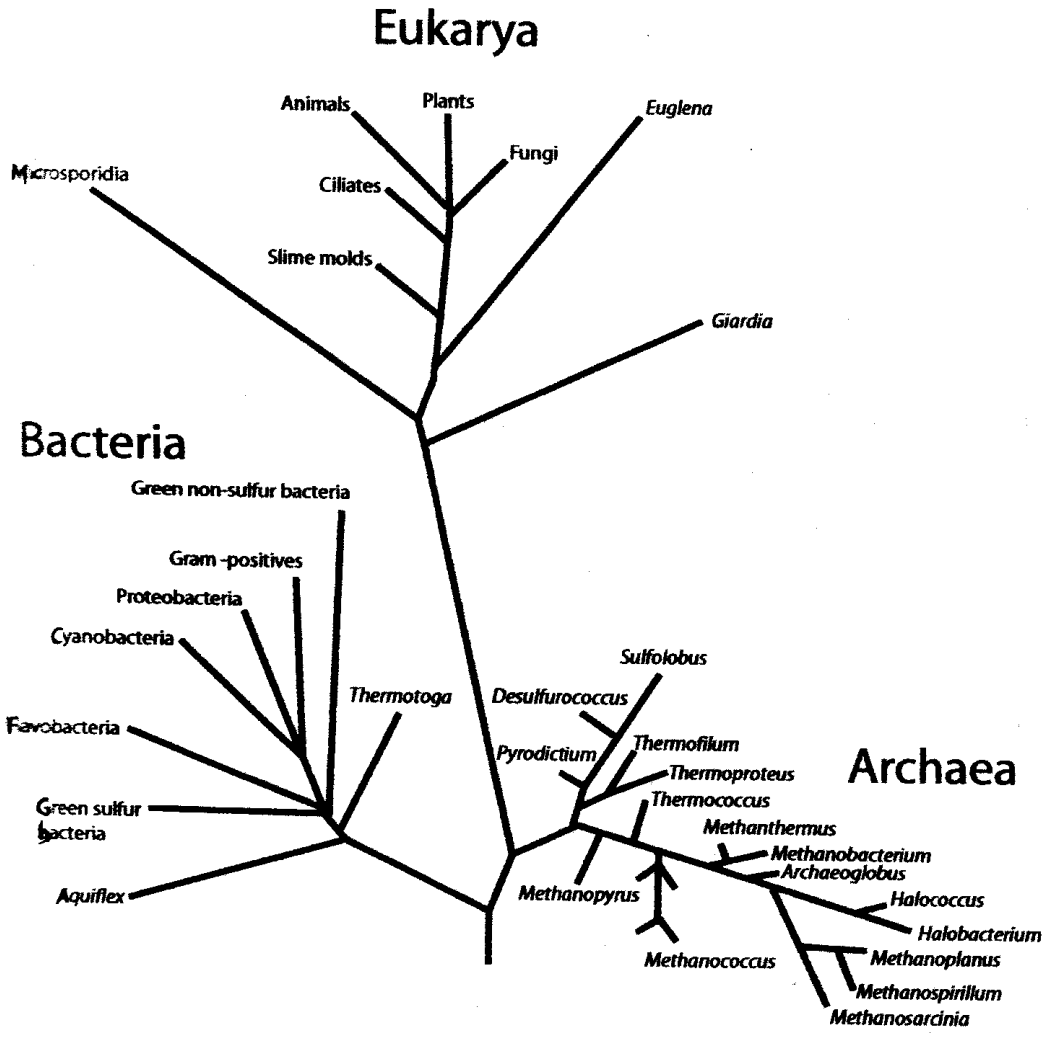

Figure 2.1. The universal phylogenetic tree of life according to Carl R. Woese (Source: Barton, 2005)

Of the two prokaryote domains, the *Bacteria* domain contains more diverse and greater number of microorganisms compared to the *Archaea* domain. Barton (2005) reported that there were 871 different genera and 5,007 species making up the *Bacteria* domain whilst there were 69 different genera and 217 species present in the *Archaea* domain. These numbers are expected to increase with increased research activities in this area. Of the two prokaryote domains, only in the *Bacteria* domain is pathogenic microorganisms found which are harmful to plants, animals and humans such as *E. coli, Salmonella, Clostrium perfringens*, *Enterococcus faecalis, Campylobacter* etc.

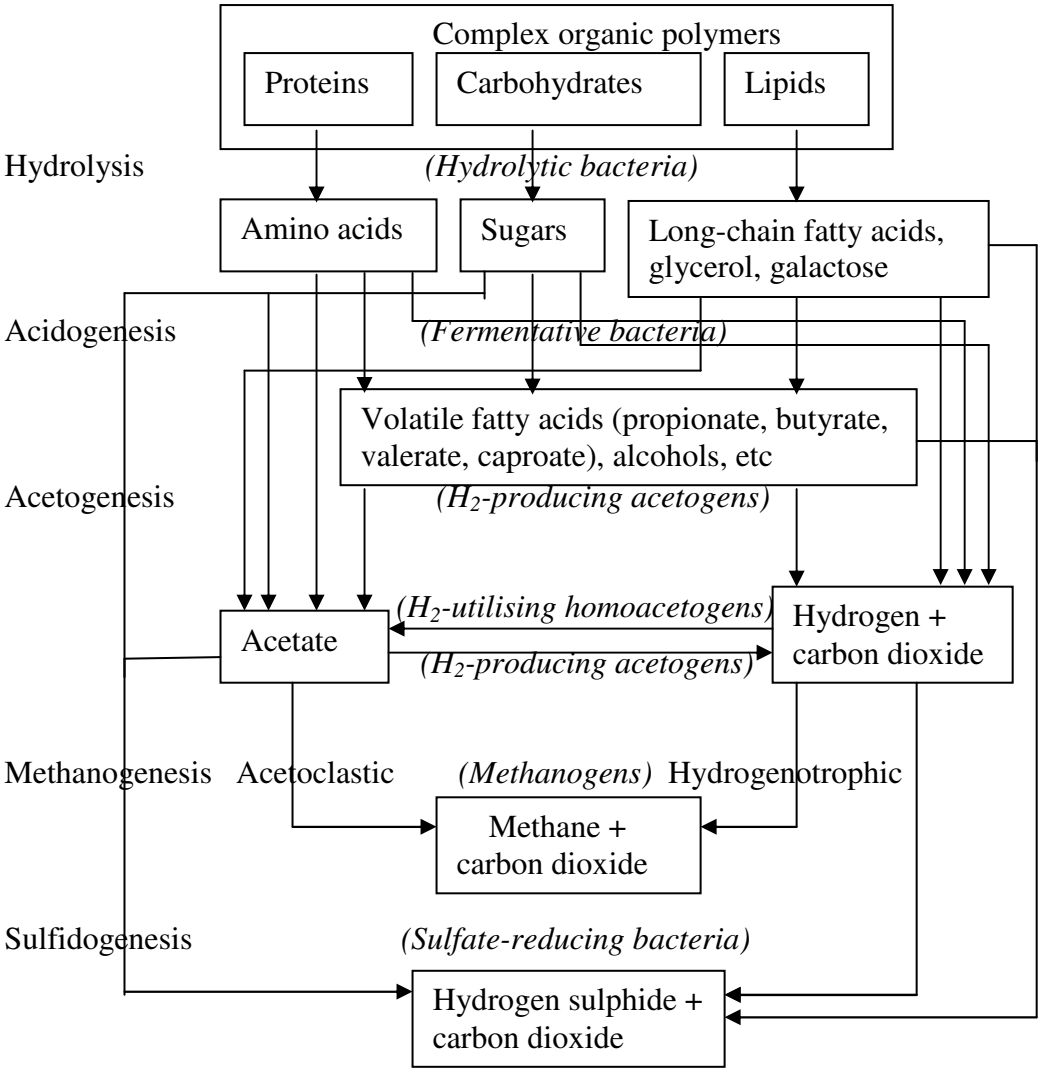

Figure 2.2. Schematic representation of the biochemical processes operating during the microbially-mediated anaerobic conversion of organic matter (adapted from de Lemos Chernicharo, 2007; Gavala *et al*., 1996; Pavlostathis and Giraldo-Gomez, 1991; and Harper and Pohland, 1986)

The anaerobic bioconversion process can be simplistically broken down into five distinct steps of hydrolysis, acidogenesis, acetogenesis, methanogenesis and sulfidogenesis (de Lemos Chernicharo, 2007). Each step is mediated by a specific prokaryotic group of microorganisms linked to their specific substrates as depicted in Figure 2.2. The microorganisms responsible for hydrolysis, acidogenesis, acetogenesis and sulfidogenesis belong to the *Bacteria* domain whilst those responsible for methanogenesis (methane production) belong to the *Archaea* domain.

However, *Aechaeoglobus* genera in the *Archaea* domain are also sulphate-reducers (Barton, 2005).

2.2.1. Hydrolysis

Hydrolysis is the first step in the anaerobic digestion process whereby the insoluble organic particulates such as carbohydrates, proteins and fats are generally first solubilised and broken down to simpler soluble materials by different exo-enzymes (eg, cellulase, amylase, xylanase for carbohydrates; protease for proteins and lipase for fats) secreted by the hydrolytic fermentative bacteria. As solubilisation is not necessarily an enzyme-catalysed biological process but could be due to physico-chemical process, hydrolysis is considered as a less defined process which is affected by several factors. These factors include reactor temperature, hydraulic retention time, substrate composition (types of carbohydrates, proteins, fats), substrate particle size, pH of the medium, enzymes production, enzyme diffusion and adsorption to substrate particles (de Lemos Chernicharo, 2007; Gavala *et al.*, 2003a). Hydrolysis is known to be a rate-limiting step in the anaerobic degradation of complex particulate substrates such as piggery waste, cattle mature and sewage (Eastman and Ferguson, 1981). As it determines the maximum substrate concentration available for the subsequent acidogenesis step at any given retention time, it would affect the overall kinetics of the anaerobic digestion process.

2.2.2. Acidogenesis (fermentation)

Hydrolysis and acidogenic fermentation can be catalysed by the same trophic group of facultative (survive in the presence of trace amount of oxygen) and obligate (survive without oxygen) anaerobic microoganisms. Amongst the anaerobic microorganisms, the acidogenic fermentative bacteria are the fastest growers with lowest doubling time of about 30 minutes (Novaes, 1986; Mosey, 1983). The predominant fermentative microorganisms that have been identified belong to the species of *Streptococcus, Bacteroids*, *Clostridium*, *Propionibacterium*, *Butyrivibrio*, *Eubacterium, Bifidobacterium, Peptostreptococcus, Peptococcus, Selenomonas, Lactobacillus* and *Enterobacteriaceae* (Banerjee *et al.*, 1998; Archer and Kirsop, 1991; Novaes, 1986). The acidogenic bacteria have an optimum growth rate in the pH range between 5 and 6 and they have a higher tolerance to lower pH values.

The soluble organic compounds generated from the hydrolysis step are taken up and metabolised by the fermentative acidogenic bacteria to simple organic compounds and excreted as volatile fatty acids (VFA), alcohols, lactic acid and mineral compounds such as carbon dioxide, hydrogen and ammonia. Table 2.1 gives some examples of products formed from the fermentation of glucose and amino acids

Table 2.1. Some examples of glucose and amino acids fermentation products

Substrate	Reaction	ΔG^o(kJ/mol)
Glucose	$C_6H_{12}O_6 + 4H_20 \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2$	-206.3
Glucose	$C_6H_{12}O_6 + 2H_20 \rightarrow CH_3CH_2CH_2COO^- + 2HCO_3^- + 3H^+ + 2H_2$	-254.8
Glucose	$C_6H_{12}O_6 + 2H_20 \rightarrow CH_3CH_2COO^- + 2HCO_3^- + 3H^+ + 2H_2$	-254.8
Amino acid	Glutamate + $3H_20 \rightarrow 2CH_3COO^- + HCO_3^- + H^+ + NH_4^+ + H_2$	-33.9
Amino acid	2 Glycine + $4H_20 \rightarrow CH_3COO^- + HCO_3^- + H^+ + NH_4^+ + H_2$	-51.5
Amino acid	Alanine + $3H_20 \rightarrow CH_3COO^- + HCO_3^- + H^+ + NH_4^+ + 2H_2$	+ 7.5
	Glycine + $2H_2 \rightarrow 2CH_3COO^- + 2\ NH_4^+$	-38.9

ΔG^o (kJ/mol) at pH 7, 1 atm, 25^{o}C

(Sources: Gallert *et al*., 1998; McInerney, 1988; Thauer *et al*., 1977)

The glucose fermentative bacteria can invoke different fermentation pathways that produce different types of metabolic products in response to environmental factors such as substrate concentration, pH and dissolved hydrogen concentration in the anaerobic digester system (Gallert and Winter, 2005; Novaes, 1986; Mosey, 1983). At low hydrogen partial pressure ($<10^{-4}$ atm), the production of acetate, hydrogen and carbon dioxide is thermodynamically favoured as this reaction provides the microorganisms with the greatest energy yield for growth. At high hydrogen partial pressures ($>10^{-4}$ atm) such as in the case of increased organic loading where hydrogen accumulates, ethanol, propionic and butyric acid are also produced via pyruvic acid in addition to acetate, hydrogen and carbon dioxide (Harper and Pohland, 1987; Novaes, 1986).

Amino acids from the hydrolysis of proteins are fermented to volatile fatty acids, ammonia, hydrogen, etc through either anaerobic oxidation linked to hydrogen production (deamination) or Stickland reaction (Table 2.1). In Stickland reaction, hydrogen is not produced as the reducing equivalents (hydrogen) produced in the oxidatively decarboxylation of amino acids such as alanine are used as electron donors to reductively convert other amino acids. An example is the conversion of

glycine to acetate and ammonia by certain proteolytic *Clostridium* species. Thus, production of hydrogen either as useful biofuel or methanogenic substrate from protein-rich substrates may prove difficult when Stickland fermentation reaction predominates. The iso-forms of VFA like iso-butyrate and iso-valerate are formed directly from reductive deamination of long-chain amino acids such as valine, leucine and i-leucine. While fermentation of sugars results in a drop in pH due to the formation of volatile fatty acids, fermentation of amino acids does not lead to a significant pH drop due to the release of ammonia. The combined ammonium ions and carbon dioxide-bicarbonate-carbonate ions provide alkalinity to counteract the volatile fatty acids produced during anaerobic digestion, thereby stabilising the wastewater pH (Gallert and Winter, 2005).

Lipids or triglycerides are hydrolysed to long chain fatty acids (LCFAs), glycerol and galactose. The soluble glycerol and galactose moities are anaerobically oxidized by fermentative acidogenic bacteria to mainly volatile fatty acids, carbon dioxide and hydrogen (McInerney, 1988). However, in contrast to sugars and amino acids, acidogenic bacteria cannot oxidise long-chain fatty acids (LCFAs) of carbon length C12 to C18 and medium-chain fatty acids of carbon length C7 to C11. Instead, these intermediate products are converted by the acetogenic bacteria via β-oxidation to acetate, hydrogen and carbon dioxide (Gurr and James, 1980).

2.2.3. Acetogenesis

Acetogenic species can be subdivided into two groups – obligate acetate-forming hydrogen-producing (proton-reducing) acetogens and acetate-forming hydrogen-utilising acetogens. Unlike the fermentative bacteria, acetate-forming hydrogen-producing acetogenic bacteria are one of the slowest growing populations, with doubling times of between 1.5 to 4 days (Novaes, 1986; Mosey, 1983). They include *Syntrophobacter wolinii* (propionate degraders) and other newer additions of propionate-degrading bacteria (de Bok *et al*., 2004) listed in Appendix 2, *Synthrophomonas wolfei* (butyrate and higher fatty acid degraders), *Syntrophus buswellii* (aromatic benzoate degraders) and sulphate-reducing *Desulfovibrio spp.* (Archer and Kirsop, 1991). *Syntrophobacter wolinii* (propionate degraders) have also been reported to take up to 7 days to regenerate (Boone and Bryant, 1980). In

contrast, acetate-forming hydrogen-utilising homoacetogens are fast growers like the fermentation bacteria, with a minimum doubling time of between 1.75 to 29 hours. They include *Acetobacterium*, *Clostridium*, *Acetoanaerobium, Acetogenium*, *Eubacterium* and *Pelobacter*. The optimum pH for the acetogenic bacteria is around 7 (Angelidaki *et al*., 2003).

The obligate hydrogen-producing acetogenic bacteria can only survive at very low concentrations of hydrogen in the environment (Stams *et al.,* 2003). Because of their strict requirement, they grow in a symbiotic relationship with hydrogenotrophic microorganisms which utilise the hydrogen produced from the anaerobic oxidation reactions of the fermentation products (volatile fatty acids, alcohols and long- and medium-chain fatty acids) by the hydrogen-producing acetogenic bacteria. Table 2.2 gives some of the anaerobic oxidation reactions of the volatile fatty acids and ethanol which are commonly observed in the anaerobic digester containing mixed anaerobic microorganisms. The end products from these reactions such as acetate, hydrogen and carbon dioxide can be directly consumed by the methanogenic *archaea* to form methane in the absence of competition from sulphate-reducing bacteria or homoacetogens (Stams *et al*., 2005).

Table 2.2. Some examples of degradation reactions of fermentation products

Substrate	Reaction	ΔG^o(kJ/mol)
Propionate	$CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + 3H_2 + CO_2$	+ 76.1
i- & n-butyrate	$CH_3(CH_2)_2COOH + 2H_2O \rightarrow 2CH_3COOH + + 2H_2$	+ 48.1
i-valerate	$CH_3(CH_2)_3COOH + 2H_2O + CO_2 \rightarrow 3CH_3COOH + H_2$	+ 20.2
n-valerate	$CH_3(CH_2)_3COOH + 2H_2O \rightarrow CH_3COOH + CH_3CH_2COOH + 2H_2$	
Caproate	$CH_3(CH_2)_4COOH + 2H_2O \rightarrow CH_3COOH + CH_3(CH_2)_2COOH + 2H_2$	+ 48.4
Caproate	$CH_3(CH_2)_4COOH + 6H_2O \rightarrow 2CH_3COOH + 8H_2 + 2CO_2$	+ 48.3
Ethanol	$CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2H_2$	+ 9.6
Hydrogen	$4H_2 + 2CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$ (homoacetogenic)	- 95.0

ΔG^o (kJ/mol) at pH 7, 1 atm, 25^{o}C

(Sources: Pind *et al*., 2002; Pohland and Kim, 2000; Thauer *et al*., 1977)

With the exception of the homoacetogenic reaction, the values of Gibbs free energy change for these reactions are positive under standard conditions (1 atm, pH 7 and 25^{o}C), indicating that they are thermodynamically not able to proceed unless the H_2 partial pressure is kept below $<10^{-4}$ atm for the reactions to be possible. These oxidation reactions are made thermodynamically favourable through inter-species hydrogen transfer, a process whereby the hydrogen released by the syntrophic acetogenic bacteria is constantly removed by the hydrogen-utilising methanogens or hydrogen-utilising sulfate-reducing bacteria (SRB) in close proximity. Examples of syntrophic acetogenic and methanogenic microoganisms are species of *Syntrophomonas* which oxidise butyrate and higher carbon fatty acids to acetate, H_2 and CO_2, and species of *Syntrophobacter* which specialise in propionate oxidation to acetate, H_2 and CO_2 in association with H_2-utilising sulfate-reducing bacteria such as *Desulfovibrio* (Boone and Bryant, 1980) or hydrogen-utilising methanogens such as *Methanobacterium* or *Methanospirillum* or homoacetogens such as *Acetobacterium, Clostridium, Acetoanaerobium, Acetogenium, Eubacterium and Pelobacter* (Archer and Kirsop, 1991; Mah 1981).

Syntrophobacter wolinii, the slowest growing propionate-converting microorganism has been shown to exhibit a far higher growth rate in co-culture with hydrogen-utilising SRB than in co-culture with hydrogen-consuming methanogens due to the higher affinity for hydrogen of the SRB in comparison with methane-producing *Archaea* (Lovley *et al*., 1982; Kristjannson *et al*., 1982). In the absence of sulphate, *Desulfovibrio* spp. are also obligate proton-reducing acetogens when metabolising ethanol or lactate whilst homoacetogens can either oxidise or synthesise acetate depending on the dissolved hydrogen concentration (Schink, 2002).

2.2.4. Methanogenesis

In the absence of other kinetically competitive biological processes, methanogenesis is the final step in the anaerobic decomposition of organic substrates whereby methane and carbon dioxide, the most reduced and oxidised forms of carbon respectively, are formed as the end products. This step is carried out by the methane-forming *Archaea* which are a highly specialised group of obligate anaerobes that utilise a limited number of substrates comprising of acetate, hydrogen + carbon

dioxide and other one-carbon compounds such as, methanol (CH_3OH), methylamines (CH_3NH_3Cl), methyl mercaptan (CH_3SH) and carbon monoxide (CO) (Gerardi, 2006; Brock *et al*., 1994). These compounds serve as direct precursors of methanogenesis.

Table 2.3 gives the methane-producing reactions from these methanogenic substrates mediated by three groups of methanogens. These groups are classified as hydrogenotrophic methanogens, methyltrophic methanogens and acetoclastic methanogens. The optimum pH for methanogens is around the neutral range of 6.8 – 7.2, although it may vary among species (Novaes, 1986).

Table 2.3. Methane-producing reactions

Substrate	Reaction	ΔG^o(kJ/mol)
Carbon dioxide/hydrogen	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	-131
Carbon monoxide	$4CO + 2H_2O \rightarrow CH_4 + 3CO_2$	-210
Formate	$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$	-304
Methanol	$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	-319
	$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	-113
Methylamine	$4CH_3NH_3Cl + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4Cl$	-230
Acetate	$CH_3COOH \rightarrow CH_4 + CO_2$	-31

ΔG^o (kJ/mol) at pH 7, 1 atm, 25^{o}C

(Source: Brock *et al*., 1994)

Hydrogenotrophic (hydrogen-utilising) methanogens produce methane from reduction of carbon dioxide by hydrogen as the electron donor. The reduction process involves a host of specific cofactors and enzymes that are unique to methanogens for the sequential reduction of CO_2 to yield methane. Most of the methanogenic species of *Methanobacterium, Methanobrevibacter, Methanothermus, Methanococcus, Methanomicrobium, Methanogenium, Methanospirillum, Methanoplanus, Methanosarcina, Methanoculleus, Methanopyrus* and *Methanocorpusculum* utilise $H_2 + CO_2$ to produce methane (Brock *et al*., 1994; Archer and Kirsop, 1991). Like the acidogens and homoacetogens, hydrogen-utilising methanogens are fast-growers, with minimum doubling time of between 4 to 11 hours (Zhang and Noike, 1991). Thus, they rapidly grow in both the acidogenic and methane reactors producing methane and carbon dioxide (Gonenc and Keresteciogl u, 1990). A minor proportion of approximately 30% of organic matter is converted to methane via the hydrogenotrophic pathway.

Methyltrophic methanogens reduce the methyl group of methyl-containing compounds to methane. The reduction process involves using part of the substrate molecules as electron acceptors and others as electron donors. Methanogenic species belonging to *Methanosarcina, Methanolobus, Methanococcus, Methanococcoides* and *Methanohalophilus* produce methane through this degradation pathway. The quantity of methane produced is relatively small in comparison to the hydrogenotrophic and acetoclastic pathways.

Acetoclastic methanogens cleave acetate to methane and carbon dioxide. Although only two genera of methanogens, *Methanosarcina* and *Methanothrix* (*Methanosaeta*) are able to carry out the acetoclastic reaction, this degradation pathway produces majority of the methane (approximately 70%) in a normal anaerobic digester. *Methanosarcina* prevail at high acetate concentration ($>10^{-3}$ M) as they have a lower affinity for acetate (Ks of 4.02 mM) but higher growth rate (μmax of 0.21 d^{-1}) compared to the strict acetoclastic *Methanosaeta* (Ks of 0.44 mM and μmax of 0.11 d^{-1}) which prevail at low acetate level ($<10^{-3}$ M). In contrast to the hydrogenotrophic methanogens, acetoclastic methanogens like the hydrogen-producing acetogens, are one of the slowest growing populations with doubling times of between 2 and 3 days (Novaes, 1986; Mosey, 1983). Thus, for efficient organic matter conversion to methane in the anaerobic digester, it is important that the environmental conditions are made favourable for the growth of the slow-growing acetoclastic methanogens as they are rate-limiting for the bioconversion of acidified wastewater to methane biogas. Both the acetoclastic and hydrogenotrophic methanogens play a pivotal role in maintaining an efficient balanced anaerobic digester. By constant removal of potential toxic metabolites such as hydrogen and acetate as they are formed, they prevent their accumulation and enhance the rate of fermentation and acetogenesis of the anaerobic digestion process (Zeikus, 1979).

Despite the large number of cultured representatives of methanogens, the cultivation-independent molecular methods of retrieval of archaeal 16S ribosomal RNA genes has shown that some species of the novel methanogenic group of rice cluster I (RC-1) are present in diverse anoxic environments but have not been culturable for isolation (Erkel *et al*., 2005).

In the presence of electron acceptors such as metal oxides [Fe(OH), MnO_2], nitrogen oxides (NO_3^-, NO_2^-), or oxidized sulfur compounds ($SO_4^=$, $SO_3^=$), methanogenesis can be inhibited and/or altered (Schink, 2006). Methanogenesis usually occurs only after these alternative electron acceptors are depleted. However, the rate of methanogenesis depends on the relative amounts of electron acceptor (e.g. acetate versus sulphate) and donor (e.g. hydrogen) present (Zeikus, 1979).

2.2.5. Sulphidogenesis

In anaerobic digesters treating wastewater containing sulfate, sulfate reduction is favourable over other reactions as sulfate-reducing bacteria (SRB) have higher affinity for the reducing equivalents or hydrogen released during the degradation of organic materials (Schink, 2006; Widdel, 1988). They are a very versatile group of microorganisms capable of using a wide range of substrate unlike the methanogens which have restricted substrate specificity. The substrates utilised by sulfate-reducing bacteria include sugars, glycerol, C2 to C18 fatty acids, ethanol, lactate and methanol as well as molecular hydrogen, aliphatic and aromatic compounds. These reactions produce hydrogen sulfide and result in a decrease in methane yield from a given amount of organic material present in the influent. Sulfate-reducing bacteria are classified into two groups - Group I non-acetate oxidizers such as *Desulfovibrio, Desulfomicrobium, Desulfobotulus, Desulfotomaculum, Desulfobulbus, Thermodesulfobacterium* and *Archaeoglobus*; and Group II acetate and C2 to C18 fatty acid oxidisers such as *Desulfobacter, Desulfobacterium, Desulfococcus, Desulfonema, Desulfosarcina* and *Desulfoarculus*; and dissimilatory sulfur-reducers such as *Desulfuromonas, Desulfurella* and *Campylobacter* (Brock *et al*., 1994).

Table 2.4 gives the competitive oxidation reactions for the methanogenic substrates by sulfate-reducing bacteria. Compared to the acetogenic (Table 2.2) and methanogenic reactions (Table 2.3), these reactions are thermodynamically more favourable as indicated by the higher negative values of the Gibbs free energy change. Thus, sulfate-reducing bacteria can easily out-compete the syntrophic consortia of acetogens and hydrogen-utilising methanogens as well as acetate-utilising methanogens (Stams *et al*., 2003, 2005; Archer and Kirsop, 1991). The

optimum pH for sulphate-reducing bacteria is in the range of 7.5 to 8.0 (van Haandel *et al*., 2006).

Table 2.4. Some examples of competitive oxidation reactions

Substrate	Reaction	ΔG^o (kJ/mol)
Propionate	$CH_3CH_2COO^- + 0.75\ SO_4^{2-} \rightarrow CH_3COO^- + HCO_3^- + 0.75\ HS^- + 0.25\ H^+$	-37.7
Propionate	$CH_3CH_2COO^- + 1.75\ SO_4^{2-} \rightarrow HCO_3^- + 1.75\ HS^- + 0.5\ H^+ + 0.25\ OH^-$	-88.9
Butyrate	$CH_3CH_2CH_2COO^- + SO_4^{2-} \rightarrow 2CH_3COO^- + HS^- + OH$	-27.8
Acetate	$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	-47.6
Hydrogen	$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	-38.1
Hydrogen	$4H_2 + SO_4^{2-} \rightarrow S^{2-} + 4\ H_2O$	-151.0

ΔG^o (kJ/mol) at pH 7, 1 atm, 25^{o}C

(Sources: Brock *et al*., 1994; Thauer *et al*., 1977)

Thus, through coupling anaerobic oxidation of reduced organic and/or inorganic compounds to the reduction of sulphate to sulphide to yield energy, SRB activity in the anaerobic reactor not only causes a reduction of methane yield, the hydrogen sulfide released can also inhibit the acetogenic and methanogenic microorganisms by removing essential trace metals as sulphide precipitates in addition to causing odour and corrosion problems to metal piping (Colleran and Pender, 2002).

2.3. REACTOR CONFIGURATIONS AND THEIR APPLICATIONS

The selection of a particular reactor for anaerobic treatment of organic wastewater is dictated by a number of factors. These include physicochemical characteristics of the wastewater, wastewater strength, treatment efficiency, reactor configuration and costs (Buitron *et al*., 2006). Aanerobic digester configurations are grouped into three broad categories: dispersed or suspended-growth, attached-growth and hybrid processes (Jordening and Buchhlz, 2005; Hall, 1992; Speece *et al*, 1997).

In suspended-growth process, the microorganisms are maintained in suspension. Examples of this process are the completely mixed tank reactor (CSTR) without and with biomass recycle (contact process). This process is well suited and mainly used for the treatment of sludge, animal manure slurry and concentrated wastewater

containing a high proportion of particulate biodegradable organic materials (Angelidaki *et al*, 2005; Hall, 1992). In the conventional CSTR without biomass recycling, the lack of retention devices to concentrate the wastewater biomass means the suspended biomass is continuously being lost through regular discharge of the treated effluent since the hydraulic retention times (HRT) and solid retention times (SRT) are the same (Halls, 1992). Thus, the effluent quality is highly dependent on the SRT (HRT) imposed on the reactor. This simple flow-through system is commonly used for the pre-acidification of wastewater with recommended HRT in the range of 6 to 24 hours (Lettinga and Hulshoff Pol, 1986). It also serves as an equalisation or holding tank to equalise the big variations in the flow or in the pollution strength of the wastewater. Table 2.5 lists the advantages and disadvantages of this configuration.

Table 2.5. Advantages and disadvantages of completely mixed suspended-growth digesters

Advantages	Disadvantages
• Suitable for wastes with high levels of suspended solids and high concentration of soluble organics. • Provide uniform substrate, temperature and pH conditions in the reactor. • Good mixing can minimise dead volume accumulation and flow channelling. • Not dependent on biomass settleability.	• Process stability may be compromised due to loss of biomass associated with short SRT. • Large reactor volume required to provide effective longer SRTs.

(Source: Hall, 1992)

An improvement to the CSTR shortcomings is the contact CSTR process which includes an external settling tank for separation and recycling of the effluent biomass to the CSTR. This allows the SRT to be controlled separately from the HRT, thereby enabling the organic loading rate to be increased and the HRT to be shortened. However, settableability of the biomass by gravity settling method can be a problem due to the biogas associated with them. Use of physical methods such as gas stripping or vacuum degasification unit or chemical method of flocculant addition in between the CSTR and settling tank will be necessary to improve the biomass settling. This is particularly important if the effluent is to be treated in a second

methanogenic reactor of a UASB system where the presence of suspended matter will impair the methanogenic performance.

In attached-growth processes which are also called fixed-film or biofilm processes, the microorganisms are attached and concentrated onto an inert medium such as sand, granular activated carbon, gravel and plastic material. Examples of these processes are fixed bed (anaerobic filters), rotating bed and expanded or fluidised bed. Because of their ability to retain a large amount of biomass, thus resulting in long solids retention times even with the application of low hydraulic retention times (<24 hr), they are commonly termed high rate wastewater treatment systems. One major limitation of the attached-growth processes is their susceptibility to clogging and hence, they are generally not suitable for particulate-containing wastewater. They are commonly used to treat dilute and soluble organic wastewater (Buitron *et al.*, 2006; Hall, 1992).

Lastly, the hybrid processes combine the beneficial features of both the suspended-growth and attached-growth processes into a single treatment package. Examples are high-rate upflow anaerobic sludge blanket (UASB), high-rate expanded granular sludge bed (EGSB), high-rate baffled reactor, low-rate anaerobic lagoon, high-rate two-stage or two-phase anaerobic systems comprising of the dispersed- and/or attached-growth reactors in series whereby the first reactor serves as the acidogenic phase with short HRT and the second reactor as the methanogenic phase with long HRT. An example of the two phase anaerobic system is the temperature-phased thermophilic and mesophilic anaerobic system (TPAD), a patented process developed at Iowa State University to meet the US faecal coliform requirement for Class A biosolid (Harikishan and Sung, 2003; Han *et al*., 1997). With the UASB process, suspended particles and precipitating matter in the wastewater can adversely affect the specific methanogenic activity of the granular sludge in the system (Lettinga, 1992). It performs best with soluble wastewater.

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Anaerobic reactors can be operated under batch, semi-continuous or continuous mode. In batch mode of operation where the substrate is fed once and let to degrade over time, steady-state conditions can never be achieved as the easily biodegradable substrate concentration decreases with time and results in the environment becoming growth-limiting to the anaerobes. In contrast, digesters with semi-continuous or

continuous operations which are drained and fed at fixed time interval or continuously respectively, allow steady-state conditions to be achieved under constant substrate concentrations, thus facilitating maximum microbial growth. Semi-continuous operation is reported to have higher substrate degradation efficiency than continuous operation due to the longer contact time between the biomass and the substrate (Budiastuti, 2004).

2.4. FACTORS INFLUENCING THE PERFORMANCE OF ACID-PHASE ANAEROBIC DIGESTION OF COMPLEX WASTEWATER

In the acid-phase anaerobic reactor where methanogenic microorganisms are intentionally inhibited or lacking, the hydrolytic and acidogenic fermentative bacteria will form an increased amount of reduced products largely as volatile fatty acids (VFA). Figure 2.3 illustrates the generalised carbon flow in the anaerobic environment without active methanogens (Ahring, 2003):

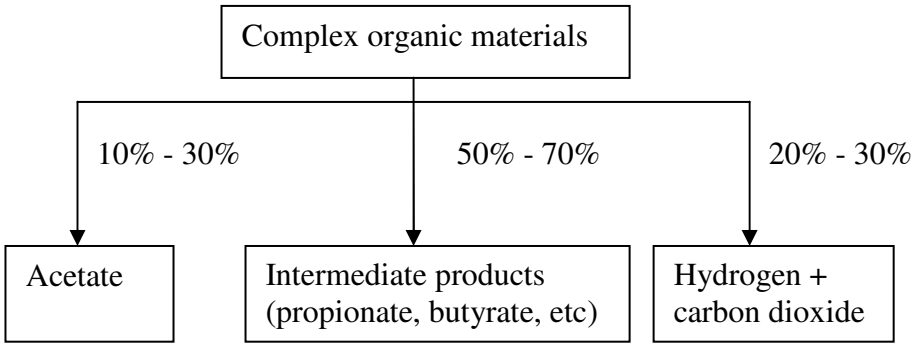

Figure 2.3. Generalised carbon flow in the anaerobic environment without methanogenesis

A number of factors can influence the types of fermentation products formed during acid phase digestion of organic waste effluents. These include wastewater characteristics; operational parameters such as hydraulic retention time (HRT) and/or solids retention time (SRT), temperature and pH (Banerjee *et al*., 1998). Knowledge of these factors facilitates one to properly engineer the acidogenic anaerobic reactors to produce metabolic intermediate products that are favourable for biodegradation by the methanogens in the methane-reactor of a two-phase anaerobic digester system.

2.4.1. Wastewater type

Wastewaters differ in the proportions of the three COD-contributing organic compounds: carbohydrates, proteins and lipids in insoluble (particulate) and soluble forms. Conversion of these organic compounds to acidified products and products distribution is influenced differently by the operational conditions imposed on the acid-phase anaerobic reactors such as pH, HRT, temperature and OLR.

Numerous studies on synthetic and real organic wastewaters have reported that the optimal conditions for acidification depend on the type of organic compounds (substrates) present in the wastewaters. Simple soluble carbohydrate substrates such as glucose (Zoetemeyer *et al.*, 1982a; Cohen *et al*., 1979), lactose (Fang and Yu, 2001; Kissalita *et al*., 1987) are readily converted to VFAs and alcohols at ≤1-day HRT and optimum pH of 5 to 5.5. Their product compositions were found not to be significantly influenced by HRT but sensitive to pH. For complex protein substrates such as gelatine (Yu and Fang, 2003) and beef extract (Dinopoulou *et al*., 1988a), product compositions were also reported not to be significantly influenced by HRT but sensitive to pH, with maximum conversion occurring at pH 6.8 to 7. For complex organic substrates containing carbohydrates, proteins and lipids, such as synthetic dairy wastewater prepared from powder milk (Hanaki *et al*., 1987; Yu and Fang, 2002) and digested primary sludge (Miron *et al*., 2000), carbohydrate was the most readily acidified while protein was acidified to a lesser degree and lipid the least acidified. Fang and Yu (2001) reported that at short HRT of 4 h, 93% of carbohydrate, 57% of protein and 20% lipid were converted to acidified forms. With increasing HRT to 24 h, protein and lipid conversions increased to 86 % and 46% respectively. Similarly with pH, Yu and Fang (2002) reported that acidification of carbohydrate increased from 95% at pH 5.5 to 98% at pH 6.5, while acidification of protein reached a maximum at pH 6.5 and 16 to 50% of lipid was acidified at pH 4.0 to 6.5.

2.4.2. pH

All enzyme-catalysed biochemical reactions are influenced by pH (McGilvery, 1983). Optimum pH for maximum hydrolysis and acidification of the complex wastewater differs for carbohydrate, protein and lipid components of the organic matter.

Hydrolysis of carbohydrate generally proceeds favourably at a slightly acidic pH while hydrolysis of protein requires a neutral or weakly alkaline pH (McInerny, 1988). For complex substrates containing a mixture of organic compounds (carbohydrates, proteins, lipid), maximum acidification of the synthetic and real complex wastewaters have been found to occur at an optimum pH range of between 5.5 and 6.3 at mesophilic temperature of 35-37^{o}C (Yu and Fang, 2002; Kasapgil *et al*., 1995; Hanaki *et al.*, 1987). At pH below 5, carbohydrate conversion was reduced by five-fold while hydrolysis of lipid to LCFA was reduced by 2-fold (Hanaki *et al*., 1987). Increasing pH from 5.5 to 6.5 produced only slight increase of conversion of carbohydrate and protein components but a two-fold increase in lipids hydrolysis. Above pH 5.5, more methane was produced with reduction in VFA concentration due to the onset of methanogenesis.

While acidification of the soluble organic matter have been extensively studied in synthetic and raw wastewaters (Yu and Fang, 2002; Inci, 1998; Kasapgil *et al*., 1995), research into the extent of hydrolysis or solubilisation of any undissolved and dissolved complex organic matter was somewhat lacking. For raw slurry-type piggery waste, Ahn *et al.* (2004) found that pH 9 was most effective in producing maximum soluble COD, ie, maximum solubilisation of the particulate organics to soluble form. Penaud *et al.* (1997) observed higher protein solubilisation of raw pharmaceutical wastewater at pH 9 (82%) than at pH 5 (6%) while carbohydrate reached its maximum solubilisation (80%) at pH 7. For municipal sludge and animal manures, several researchers had successfully employed alkaline chemical pre-treatment either singly (Angelidaki and Ahring, 2000; Lin *et al*., 1997) or in combination with physical methods such as thermal, ultrasonic, ball milling and maceration (Lee *et al*., 2009; Liu *et al*., 2009; Delgenes *et al*., 2000; Chiu *et al*., 1997) to enhance the solubilisation of particulate organic materials.

2.4.3. Temperature

Anaerobic treatment of organic wastes and wastewaters is commonly carried out at the optimum mesophilic temperature of 35-37^{o}C and thermophilic temperature of 55-60^{o}C. For temperatures in-between these two optimum temperatures, studies on the performance of fermentative microorganisms in acidifying the organic substrates

appear to produce contradictory findings. Zoetemeyer *et al.* (1982a) found that the acidogenesis performance of acid-phase CSTR on glucose substrate dropped outside the optimum mesophilic and thermophilic temperature ranges of 36-38^{o}C and 51-53^{o}C respectively at pH 5.8 and HRT of 1-2 hr. Banerjee *et al.* (1998) also found VFA production from mixed industrial and municipal wastewater to be optimum at a lower mesophilic temperature of 30^{o}C but dropped by 35% at 35^{o}C in the mesophilic acid-phase CSTR. In contrast, Yu and Fang (2003) and Fang and Yu (2001) observed increased acidification of gelatine and lactose respectively increased with temperature from 20 to 55^{o}C when treated in upflow acid-phase reactors at HRT of 12 hr and pH 5.5.

Process stability of thermophilic anaerobic digester is another conjecture area amongst researchers. Zoetemeyer *et al.* (1982a) reported that at high organic loadings, acid-phase anaerobic CSTR reactor was more stable at mesophilic than at thermophilic conditions. Yu *et al.* (2002a) on the other hand, noticed that both the mesophilic and thermophilic upflow reactors showed similar propionic acid levels at high organic loading of synthetic dairy wastewater at pH 5.5 and HRT of 12 h, which indicated that the stability of thermophilic reactor was comparable to that of mesophilic reactor.

Thermophilic temperature is widely known to be more effective in pathogen destruction than mesophilic temperature (Salsali *et al*., 2008; Bendixen, 1994). However, findings on the acidification of organics were often contradictory (Yu *et al*, 2002a; Pavan *et al*., 2000; Guerrero *et al*., 1999). Other disputed area is with regards to the benefits of operating anaerobic reactors at thermophilic condition versus mesophilic condition. Some researchers reported that the first-phase acid reactors of a two-phase anaerobic digester (Ahn *et al*., 2004; Yu and Fang, 2003; Yu *et al*., 2002a) showed comparable performance in organics acidification at thermophilic and mesophilic conditions. Yet other researchers reported that thermophilic acid-phase reactors had higher performance in terms of the extent of solubilisation (Kuang, 2002; Guerrero *et al*., 1999) and acidification (Ahn and Forster, 2002b; Guerrero *et al*., 1999) of the organic matter as well as effective pathogens destruction (Bendixen, 1994; Engeli *et al.,* 1993; Duarte *et al.,* 1992; Mitsdörffer *et al.,* 1990; Lee *et al.,* 1989) compared to mesophilic acid-phase reactors.

2.4.4. Hydraulic retention time (HRT)

Hydraulic retention time (HRT) is one of the many process parameters that affect the microbial performance of the hydrolysis-acidification reactor as it determines the extent of achievable hydrolysis of particulate organic matter and acidification of the soluble organic components (Guerrero *et al*., 1999; Alexiou *et al*., 1994). The first acid-phase reactor of a two-phase anaerobic system is normally operated at a very short HRT of between 6 and 24 hours to purge out the slow-growing methane-producing microorganisms (Lettinga and Hulshoff Pol, 1986). However, it is unclear whether such short operating HRTs would be effective in solubilising the particulate organic matter presents in complex organic wastewaters such as from the livestock and food processing industries which typically contain undissolved and dissolved organic components made up of a mixture of carbohydrates, proteins and lipids.

With synthetic dairy wastewater, Hanaki *et al.* (1987) found complete hydrolysis and acidification of carbohydrate at HRT >6 hr while hydrolysis of protein required longer HRT of 30 hr to reach about 60% at pH 6-7. Net acid production also increased with increasing HRT while methane production also increased. Guerrero *et al.* (1999) reported that maximum VSS removal and acidification efficiencies of fish meal processing wastewater occurred at 1-day HRT at 55^{o}C (58%) and 37^{o}C (46%). With synthetic and raw dairy wastewater, maximum acidification occurred at 12-hr HRT (Demirel and Yenigun, 2004; Fang and Yu, 2001). For animal wastes, it is observed that HRT of more than 1 day is widely applied on the first-phase reactor of a two-phase anaerobic system. Cseh *et al*. (1984) applied long HRT of 2 to 4-days to treat liquid swine waste with dry matter of 2.5 to 7% at pH around 6.4. Ahn *et al.* (2004) too adopted a long HRT of 5-day and alkaline pH of 9 for the treatment of raw slurry-type swine waste with total solids of around 2.0%. Harikishan and Sung (2003) adopted 4-day HRT for the first-phase thermophilic (55^{o}C) treatment of raw cattle manure to enhance hydrolysis of the recalcitrant organics and to destroy pathogenic organisms while Demirer and Chen (2005) applied 2-day HRT for the first-phase mesophilic treatment of unscreened dairy manure. Despite the variation in the applied HRT, methane production was reported in all cases irrespective of the loading rates which suggested that complete phase separation of the acidogenesis and methanogenesis was impossible to achieve.

2.4.5. Organic loading rate (OLR) or substrate concentration

Several studies have demonstrated that OLR or substrate concentration affects the extent of acidification and the acidified product distribution (Kim *et al*., 2004; Yu and Fang, 2001; Yu *et al.,* 2002; Inci, 1998). Acidification tends to increase up to a certain OLR or substrate concentration and then decrease beyond it. Kim *et al.* (2004b) working on synthetic wastewater containing long-chain fatty acids and glucose observed increased organic conversion to VFAs, hydrogen (0.1-0.7%) and methane (30-50%) from 1 to 5 g COD/L/d at pH of around 7 and HRT of 18 hr. Beyond 5 g COD/L/d, methane production rate and LCFA acidification efficiency dropped while hydrogen production increased. Similarly, Yu and Fang (2001) and Yu *et al.* (2002a) reported a drop in the extent of acidification of synthetic dairy wastewater from 61% to 27% as the COD loading rate increased from 2 to 30 g/L/d in both the thermophilic and mesophilic UASB acidogenic reactors at 12-h HRT. Carbohydrate was the most easily acidified organic compound at all OLR, followed by protein. Product distribution showed acetate and butyrate decreased while propionate, propanol, butanol and biogas production rate increased with increasing influent COD. Low influent COD strength yielded biogas with low level of hydrogen and some 15% of methane while high influent COD strength yielded biogas with increase level of hydrogen and no methane. Inci (1998) reported that the distribution and concentrations of the four main VFAs (acetic, propionic, n-butyric and n-valeric acids) changed with the OLR of dairy wastewater. Acetic and propionic acids were detected up to an OLR of 4 g COD/L/d but n-valeric acid was predominant as OLR increased to 23 g COD/L/d, followed by n-butyric, acetic and propionic acids.

2.4.6. Toxicants or inhibitors

Hydrogen affects substrate conversion potential of almost all major anaerobic bacteria groups in the anaerobic treatment process (Harper and Pohland, 1986). Molecular hydrogen is released alongside the excreted reduced metabolic products by fermentative bacteria during acidogenesis of the organics. Under high substrate load or inhibition of hydrogen-utilising bacteria which gives rise to high hydrogen partial pressure of $>10^{-4}$ atm, accumulation of propionic and butyric acids occurs. These products can in turn inhibit their own anaerobic oxidation through product

inhibition mechanism, thereby stalling the digestion process. The unfavourable thermodynamic and kinetic conditions in the acid-phase reactors with low HRT (<24 hr) and high partial H_2 partial pressure (>0.04 atm) explains why lipid degradation hardly occurred in acid-phase reactors as the growth of syntrophic LCFA-degrading acetogens and methanogens which convert LCFAs to shorter-chain VFAs were inhibited (Fox and Pohland, 1994; Hanaki *et al*., 1987).

Partial acidification (20-50%) as oppose to complete acidification of the organic wastewater in the acidogenic reactor is recommended by most researchers as highly acidified wastewater can cause product inhibition on the acidogenic bacteria and adversely affect the methanogenic reactor of UASB configuration (Hwang *et al*., 2001; Alexiou *et al.,* 1994; Lettinga and Hulshoff Pol, 1986; Dinopoulou *et al*., 1988a, 1988b; Zoetemeyer *et al.* 1982a). Zoetemeyer *et al*. (1982b) studied product inhibition by volatile fatty acids on glucose acidogenesis in a mesophilic CSTR at pH 5.5 and HRT of 4 hrs. At butyrate input concentrations above 1700 mg/L, glucose acidification showed increasing inhibition in the form of decrease biogas production (H_2 and CO_2) and increase glucose level in the effluent.

In Demirel and Yenigun's (2002) review of the studies on inhibitory/toxic effect of heavy metals on acid-phase anaerobic reactor, copper at 1-10 mg/L was reported to be most toxic to VFA-producing microorganisms compared to zinc at 5-40 mg/L and lead. Between cadmium and nickel, cadmium at dosages over 20 mg/L was more toxic while chromium at dosages over 5 mg/L was more toxic than cadmium on acidogenic bacteria. For organic substances, pentachlorophenol concentrations up to 35 mg/L partially inhibited the acidification of glucose in the acidogenic reactor.

2.4.7. Seed types

Proper acclimatization of sludge with the intended substrate is widely recognized to be important for the selection and enrichment of appropriate microbial populations for maximum substrate utilization and degradation kinetics. Gavala and Lyberatos (2001) demonstrated this aspect using lactose- and gelatin-acclimatised cultures on various organic compounds. When the cultures were fed with glucose and/or lactate, substrate utilisations were fast for lactose-acclimatised culture as a result of indirect acclimatisation to lactose hydrolysis products during the acclimatisation period. In

contrast, gelatin-acclimatised culture required a considerable long adaptation period prior to the degradation of the two substrates due to the need to develop the appropriate enzymes as glucose and lactose were not the intermediate products of gelatin (protein) biodegradation. Similarly, Perle *et al.* (1995) showed that solubilisation of protein was much faster by casein-acclimatised sludge than the unacclimatised sludge due to the presence of the proteolytic enzyme.

2.5. FACTORS AFFECTING THE PERFORMANCE OF SINGLE-STAGE ANAEROBIC DIGESTION OF COMPLEX WASTEWATER

Conventional single-stage anaerobic digestion is susceptible to process upset due to the sensitivities of methanogenic microorganisms in particular, to changes in the environmental conditions. Factors that affect and regulate the digester's performance in degrading complex wastewater to methane and carbon dioxide include the following:

2.5.1. pH and buffering capacity

Methane-producing *archaea* are active within the pH range of 6.8 to 7.2 (Novaes, 1986). With decreasing pH, methane-producing *archaea* become more inhibited whilst the fermentative bacteria become more active, even at pH 4.5 and produce more fatty acids. These acids can overcome the wastewater alkalinity and depress its pH, causing inhibition of the methanogens. Changes in wastewater pH in the anaerobic digester alter the chemical form of waste compounds to ionised or non-ionised form and hence their toxicity to the microorgansims (Geraldi, 2006). For example, the non-ionised/undissociated form of ammonia (NH_3) and hydrogen sulphide (H_2S) are more toxic than their ionised forms (NH_4^+) and HS^- as the non-ionised forms diffuse through the cell membrane of the microorganisms more rapidly. The buffering capacity or alkalinity of a biological system is shown by its degree of resistance to changes in pH. Alkalinity in the anaerobic digester is derived from the degradation of organic-nitrogen compounds, such as amino acids and proteins, and the production of carbon dioxide from the degradation of organic compounds. When amino acids and proteins are degraded, amino groups ($-NH_2$) are released to form ammonia (McInerney, 1988). The ammonia dissolves in water along with CO_2 to

form ammonium bicarbonate ($NH_4HCO_3^-$) which contributes to the alkalinity content. Alkalinity is important for process stability as it serves to neuralise the organic acids produced during organic degradation.

2.5.2. Temperature

Microbial growth rates are strongly temperature-dependent, with growth rates increasing with temperature up to 60^{o}C (Pohland and Ghosh, 1971). Temperature also influences the physical properties of the wastewater such as viscosity, surface tension and mass transfer. In general, anaerobic reactors are normally operated at mesophilic (30 to 40^{o}C) or at thermophilic temperature (50 to 60^{o}C). Higher temperatures can result in instability of the reactors as a result of VFAs accumulation in the effluent while under psychrophilic temperatures (below 25^{o}C), the process rates are slowest compared to mesophilic and thermophilic temperatures. As with the acid-phase anaerobic digestion, two optimal temperature ranges, one at mesophilic (around 35^{o}C) and the other at thermophilic (55 to 60^{o}C) temperatures with decreasing rates between these two optima, have often been cited. However, Macki and Bryant (1981) suggested that the low rates between these two optima might have been due to a lack of adaptation.

Anaerobic waste digestion at thermophilic temperature is reported to be more efficient than at mesophilic temperature in that it increases the reaction rates of particulate hydrolysis yielding higher degradation rate, higher organic loading rates, shorter effective HRT or SRT, smaller reactor volume required to treat the same quantity of waste, greater temperature shock resilience and more importantly, greater reduction of pathogens (Angelidaki *et al*., 2003; Ahn and Forster, 2002a; 2002b; Guerrero *et al*., 1999; Bendixen 1994; van Lier *et al*., 1994; Lee *et al*., 1989). Smith *et al.* (2005) found that *Escherichia coli* and *Salmonella* spp. are not destroyed by mesophilic temperatures, whereas rapid inactivation occurs by thermophilic digestion. Some researchers however, reported that thermophilic anaerobic process was less stable to environmental changes and more favourable for the production of propionate than the mesophilic process (Fang and Chung, 1999; Wiegant *et al*., 1986; Zoetemeyer *et al*., 1982a). Others found no improved performance in organic substrate degradation with the thermophilic reactors compared to mesophilic reactors

(Yu *et al*., 2002; Wiegant *et al*., 1985; Fang and Chung, 1999). These contradictory findings between researchers besides the higher energy requirement to heat thermophilic reactors may have contributed to thermophilic anaerobic reactors being less popular than their mesophilic counterparts.

2.5.3. Hydrogen sulfide

Hydrogen sulphide is produced in the anaerobic digester through the reduction of sulphate (SO_4^{2-}) and the fermentation of sulphur-containing proteins. Undissociated H_2S is one of the most toxic compounds to methane-producing *Archaea* and also sulphate-reducing bacteria as it combines with the iron of cytochromes (electrons transfer proteins) and other essential iron-containing compounds in the microbial cells, causing the electron transport system to cease functioning (Brock *et al*., 1994). It has been reported that total hydrogen sulphide concentrations of 100 to 300 mg/L or free H_2S of 50 to 150 mg/L caused severe inhibition and eventual complete cessation of the biogas production. Gallert and Winter (2005) reported that at a total sulphide concentration of 270 mg/L, 50% of methanogenesis was inhibited while a lower concentration of 85 mg/L inhibited sulphate-reducing bacteria. In anaerobic digester treating waste with high ammonia concentration such as animal manure, a lower sulfide concentration of 23 mg/L was reported to reduce methane production by some 40% (Hansen *et al*., 1999). The presence of large amount of H_2S can precipitate essential heavy metal ions, such as iron, nickel, molybdenum, cobalt etc as metal sulfides which then lead to deficiencies in heavy metal bioavailability for the methanogens.

Figure 2.4 shows the influence of pH and temperature on the dissociation of hydrogen sulphide and ammonia. At low pH values and low temperature, formation of undissociated hydrogen sulfide is favoured over the ionised sulphide form (Stams *et al.,* 2005, 2003).

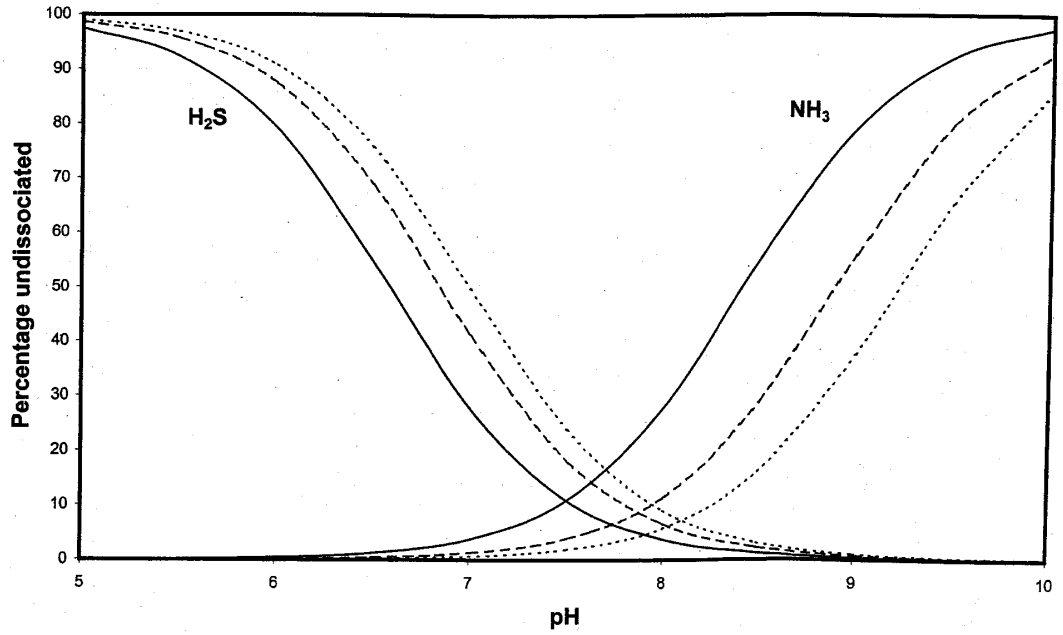

Figure 2.4. Effects of pH and temperature on the dissociation of hydrogen sulfide and ammonia (….. 25°C, ------ 37°C, —— 60°C) (Source: Stams *et al*., 2003)

Sulfide toxicity can be minimised by increasing the pH, addition of iron salts to precipitate sulphide from solution, biomass recycling to select sulphide-tolerant microorganisms and diluting digester feed (de Lemos Chernicharo, 2007; Hall, 1992).

2.5.4. Ammonia

Organic wastes such as pig, cattle and poultry manures, slaughterhouse waste, organic fraction of municipal solid waste, seafood waste and highly proteinacous wastewater often contain high concentrations of toxic ammonia (Calli *et al*., 2005; Angelidaki *et al.,* 2003). Ammonia is considered a major inhibitor of the methanogenesis process in anaerobic digesters treating animal wastes and is the primary factor limiting the organic loading rate (Hashimoto, 1983). Ammonia is produced in the anaerobic digester through the fermentation of amino acids and nitrogen-containing compounds such as urea. It is widely known that the non-ionised form of dissolved ammonia is responsible for inhibition in the biogas process (Gallert *et al*., 1998). McCarty (1964) reported that ammonia inhibition occurred at total ammonia (free ammonia plus ammonium) concentrations between 1.5 and 3.0 g/L N at pH above 7.4 in the mesophilic range whilst ammonia toxicity occurred at concentrations above 3.0 g/L at all pH levels. However, the inhibitory level of free

ammonia depends strongly on the acclimatisation of biomass to ammonia. Angelidaki and Ahring (1994) observed that the biogas process could be adapted to tolerate free ammonia concentration of 800 mg-N/L. In contrast to hydrogen sulfide, Figure 2.4 shows that as free ammonia concentration increases with increasing temperature and pH, more free ammonia concentration is present at thermophilic than at mesophilic temperatures. Means to reduce ammonia toxicity include diluting the digester feed, reducing the pH, gradual acclimatisation of the biomass and treatment with ion exchanger such as zeolite (Kotsopoulos *et al*., 2008; Tada *et al*., 2005; Milan *et al*., 2001; 2003; Sánchez *et al*., 1995; Yadvika *et al*., 2004).

2.5.5. Long-chain fatty acids

Wastewaters from slaughter houses, dairy and vegetable oil processing contain lipids that can cause toxicity in anaerobic digesters. Fats are esters of glycerol and long-chain fatty acids (LCFA) which are released during fermentation (Gurr and James, 1980). Unsaturated LCFAs such as oleic acid (C18:1) and linoleic acid (C18:2) have greater inhibitory effect than saturated LCFA such as stearic acid (C18:0) and palmitic acid (C16:0) on methane production from acetate and hydrogen as well as acetogenic beta-oxidation of LCFAs (Komatsu *et al.,* 1991; Hanaki *et al.,* 1987). Komatsu *et al*. (1991) and Hanaki *et al*. (1987) found that the inhibitory effect of unsaturated long-chain fatty acids (LCFAs) could be minimised through saturation (hydrogenation) of the unsaturated LCFAs. Lower pH was less effective in converting unsaturated oleate to the non-inhibitory saturated palmitate.

2.5.6. Organic overload

Organic overloading through either increasing feed concentration at a given HRT or decreasing the HRT at a given feed concentration will occur when the organic influent to the reactor exceeds the bioconversion capability of the microbial ecosystem. Organic overloading tends to occur in anaerobic reactors treating intermediate to high concentrations of the soluble and easily biodegradable organic substrates that have low buffering capacity such as carbohydrates-rich wastes from sugar, starch, potato, distilleries and fruit processing factories.

During organic overloading, hydrogen which is released by the fermentative bacteria from glucose degradation accumulates and results in an elevated hydrogen partial pressure (>10^{-4} atm) and a shift in the fermentative pathway from acetate, hydrogen and carbon dioxide production to ethanol and longer-chain VFAs such as propionic, butyric acid and valeric acid (Gallert and Winter, 2005; Harper and Pohland, 1987). These products in high concentration can cause inhibition to the methanogenic *Archaea*. In the case of organic overloading caused by HRT reduction, accumulation of VFAs occurs when the HRT was shorter than the generation time of the slow-growing VFA-degrading acetogenic bacteria and acetoclastic methanogens as observed in the acid-phase anaerobic reactors with very short HRT of less than 24 hr (Yu and Fang, 2001; Zoetemeyer *et al.* 1982a).

2.5.7. Hydrogen

Thermodynamics play an important role in the degradation of various organic compounds (Harper and Pohland, 1986: Thauer *et al*., 1977). All oxidation-reduction reactions are described in thermodynamic terms by the biochemical standard Gibbs free energy change values (ΔG) of the substrate catabolism at standard conditions of pH 7, 1 atm. and 1 kg/mol activity. The overall redox reaction is energetically favourable only if the net free energy change ΔG has a negative value. Anaerobic oxidation reactions of the VFAs and alcohol shown in Table 2.2 of Chapter 2 are thermodynamically possible only when the reduced product (hydrogen) is maintained at low concentrations by the scavenging activities of the hydrogen-utilising microorganisms. Thus, increasing the biological hydrogen removal capacity of the reactor via hydrogen-utilising microorganisms such as methanogens, sulfate-reducing and nitrate-reducing bacteria, and/or the physical hydrogen removal capacity through gas release are means to maintain hydrogen levels below 10^{-4} atm (10^{-8} M) so as to allow the continuous and efficient oxidation of propionic and higher organic acids (Fox and Pohland, 1994; Harper and Pohland, 1986).

2.5.8. Macro-nutrients, trace and heavy metals

As methanogenesis by either the acetate- or hydrogen-utilising methanogenic *Archaea* is the rate-limiting step in the anaerobic digestion of soluble wastes, it is

therefore not surprising that research attention has primarily focused on the nutritional requirements for the growth of methane-producing *Archaea* with the aim to optimize the overall anaerobic digestion process. Macro-nutrients such as carbon, nitrogen, hydrogen, sulfur, potassium, phosphorus, calcium and magnesium are required at concentrations ranging from 1 to 10mM concentrations (Archer and Kirsop, 1991). Trace metals such as cobalt, iron, nickel, selenium and molybdenum are also required for co-enzymes and enzymes involved in essential methanogenesis (Mulrooney and Hausinger, 2003; Ragsdale, 1998; Brock *et al*., 1994). For example, nickel is a key component of important methanogenic coenzymes such as F_{420} and F_{430} which are present in all methanogenic microorganisms. It is also present in the enzymes dehydrogenases and acetyl-coenzyme A decarbonylase/synthase. Iron, cobalt, selenium and molybdenum are also present in many of the anaerobic microbial enzymes. Heavy metals such as nickel, copper, lead and zinc are toxic to the anaerobes at relatively low concentrations.

2.6. NUCLEIC ACID-BASED MOLECULAR METHOD FOR MICROBIAL ANALYSIS

Prior to the development and establishment of advanced molecular biological techniques, traditional cultivation-based methods such as dilution plating and most probable number (MPN) estimates of anaerobic bacteria on selective enrichment culture media were common techniques used to isolate, identify and enumerate the different types of viable microbial populations present in wastewater and environmental samples. They have also been used to evaluate the sanitisation efficiencies of waste and wastewater treatment systems based on selected indicator bacteria such as faecal coliforms and intestinal enterococci (faecal streptococci). The major limitations of these traditional methods are that they are slow, laborious and they have been shown to substantially under-estimate the complex microbial populations due to their biases in selecting a small fraction of those that can be cultivated on the chosen media whilst majority of the uncultivated viable communities were not selected (Lebuhn *et al*., 2005; Amann and Ludwig, 2000).

Since the mid-1980s, several molecular biological methods involving 16S-rRNA or rDNA gene probing and sequencing such as fluorescence *In Situ* hybridisation

(FISH), polymerase chain reactions (PCR) and community profiling methods such as terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) or combinations of PCR-amplified 16S-rDNA and DGGE (PCR-DGGE), TGGE ((PCR-TGGE) or T-RFLP (PCR-TRFLP) have gradually evolved and established as powerful molecular tools available to assess the diversity, abundance and distribution of microorganisms in natural ecosystems and wastewater treatment systems (Erkel *et al*., 2005; Diaz *et al*., 2003; Rittmann, 2002; Head *et al*., 1998). These cultivation-independent methods have overcome many of the limitations of the traditional cultivation methods. They are faster and more reliable than the cultivation methods and the genetic sequences can be obtained directly from the environment without prior cultivation of the organism of interest.

Consequently, they have now been extensively applied for *in-situ* identification and quantification of specific microbial groups which included the uncultivated microorganisms at different phylogenetic levels in anaerobic treatment processes (Amann *et al*., 1997, 1995). Despite the advantages of these molecular approaches, they lack the capability to provide direct information on the physiological and functional characteristics, particularly of the uncultivated and hence unidentifiable microbial populations in contrast to the pure culture method which can provide in-depth information. Thus, the molecular methods should be regarded as useful complements rather than substitutes of the traditional cultivation methods as the latter are still useful for the enrichment and isolation of microbes of interest that have been identified by the molecular-based method through using various selective organic substrates as energy and carbon source (Erkel *et al*., 2005; Rittmann, 2002; Sekiguchi *et al.,* 2002). Using this enrichment cultivation-based, goal-directed approach, Sekiguchi *et al.* (2002) successfully isolated a total of five new species of thermophilic anaerobic microorganisms and a number of bacteria which were detected by the molecular-based methods.

Whole cell fluorescence *in situ* hybridisation (FISH) method involves the use of fluorescence-labelled group- or species-specific oligonucleotide probes to hybridise to the targeted sequences of evolutionarily conserved or variable nucleotides of the ribosomal RNA (rRNAs) within the intact microbial cells under optimum

hybridisation conditions (Amann *et al*., 1990). Unlike rDNA, rRNA is present in abundance in every living cell and hence they can be detected directly (Barton, 2005). RNA synthesis does not require considerable energy and it plays a critical role in protein synthesis. Its small subunit (SSU) rRNA, also known as 16S rRNA for *Bacteria* and *Archaea*, has approximately 1500 bases or nucleotides that can be sequenced (Rittmann, 2002). Oligonucleotide probes (small DNA molecules of between 15 and 20 bases) are designed to perfectly complement the targeted sequences in the 16S rRNA. They are commonly labelled with fluorochrome to enable microbial visualization under fluorescence microscopy.

By selecting the appropriate rRNA-targeted probes, FISH can be used to detect all bacteria cells using universal domain-specific probes such as Eubmix probe for *Bacteria* and Arc probe for methanogenic *Archaea* or a single population of cells such as a group-specific probe that can identify members of larger phylogenetic bacteria groups such as the alpha-, beta-, gamma- subclasses of Proteobacteria, Gram-positive bacteria with a high or low DNA G+C content, or *Cytophaga-Flavobacterium* cluster. Thus, they yield important data on the abundance of different phylogenetic groups in different environments. Table 2.6 outlines the strengths and weaknesses of the molecular FISH method.

In contrast to FISH, polymerase chain reaction (PCR) method targets the rDNA which are present in small quantity in the cells and hence, cannot be detected directly. They are amplified or replicated up to 10^6 fold or more through the use of selective primers to produce more DNA copies for easier microbial detection and characterisation (Scragg, 2005; Rittmann, 2002). The primers are oligonucleotides that have a sequence complementary to the target single strand DNA (ssDNA) template for amplification. The advantages of this method over the traditional cultivation-based methods are that it is relatively fast and easy to get results within 24 hours. Its disadvantages are that it is qualitative and it includes all viable (live) and non-viable (dead) cells. Lebuhn *et al.* (2005) agree that even the new powerful molecular technique of quantitative real-time PCR (qPCR) which quantifies specific microorganism based on their gene copy numbers, suffers from the same disadvantage of including DNA genes from non-viable or decaying cells, resulting in considerably high results on pathogen populations in the anaerobic digester-treated

wastewater. In such doubtful situations which can cause false alarm, they recommend that a supplementary method that detects only viable cells be carried out to verify the PCR-based microbial results.

When used in combination with molecular fingerprinting techniques such as T-RFLP, DGGE or TGGE, population dynamics at species level can be determined from the electrophoretically separated amplified DNA which are visualised as characteristic bands that correspond to different microorganisms. The individual bands can be cut from the gels, sequenced to identify the species that constitute the microbial community and their proportions quantified using FISH method (Kurisu *et al.*, 2002). T-RFLP analysis involves using restriction enzymes to degrade the extracted DNA or PCR-amplified DNA to yield a specific number of DNA fragment bands after gel electrophoresis. DGGE and TGGE methods involve changes in the electrophoresis mobility of different DNA fragments migrating in a gel containing linearly increasing gradient of DNA denaturants such as urea/formamide or temperature respectively (Scragg, 2005).

2.7. CONCLUSION

This literature review highlights the great complexities associated with anaerobic treatment of complex organic wastewater. The type of reactor used, wastewater composition, microbial composition and reactor operating conditions, in particular pH, temperature, HRT, OLR and substrate concentration all exert significant influence on the extent of organic substrates conversion to intermediate metabolic products in the first-stage acidgonic reactor or degradation to methane and carbon dioxide in the second-stage methanogenic reactor or single-stage reactor. Intermediate metabolic products such as ammonia, hydrogen, VFAs formed during the organics conversion can become toxic to the anaerobic microorganisms above certain threshold levels. The latter are largely dependent on organic substrate concentration or OLR, pH and temperature of the reactor.

Table 2.6. Strengths and weaknesses of FISH method compared to traditional cultivation-based methods

Strengths	Weaknesses
• No cultivation of the microoorganisms required. • Microbial cells can be preserved with fixatives and frozen for future analysis after cell pellet was washed and suspended in phosphate buffer-ethanol. • More reliable as it includes all culturable and unculturable microorganisms. Allows direct visualisation of non-cultured microorganisms. • It allows identification and quantification of the proportion of microbial groups of interest, from domain-level down to species-level in their natural spatial position (if the probes have been designed and available). • Detects viable (live) cells only. • Simultaneous hybridisation with two or three differently labelled probes provides a mean to check whether 'cross-hybridising' populations are present in the sample. • Ideal for visualising the spatial distribution and activity of microorganisms within the biofilm and sludge flocs.	• Cell counts using FISH may be underestimated by the lack of equipment sensitivity for visualization of all cells or problem with probe permeability of the cells, in particular the Gram-positive *Bacteria*. • It may hybridise with as yet unknown microorganisms which are phylogenetic members of a probe-targeted group, but do not contain a perfectly matching target site. • Quantification can be tedious, time-consuming and subjective with manual counting or complex with image analysis equipment - requires personnel judgement and experience. • Structural analysis of aggregates (granular sludge, biofilms) requires trained personnel and expensive equipment such as confocal microscope and image analysis devices.

(Sources: Sanz and Köchling, 2007; Kurisu *et al*., 2002; Amann *et al*., 1997, 1995)

CHAPTER 3
GENERAL MATERIALS AND METHODS

3.1. INTRODUCTION

This chapter covers the general reactors set-up for the batch acidogenic and the semi-continuous experiments as well as the general analytical methods used in the preparation and testing of samples. Materials and methods specific to the particular experiments are given in their respective chapters.

3.2. REACTORS DESIGN AND SYNTHETIC COMPLEX WASTEWATER

3.2.1. Acidogenic culture reactors

Three insulated 3-L Schott bottles with heating tapes wrapped round them were used as the acidogenic reactors to cultivate acidogenic bacteria at 37^{o}C, 47^{o}C and 55^{o}C for use in the batch vial and semi-continuous reactor experiments on synthetic complex wastewater. The three temperatures were chosen to allow solubilisation and acidification performances of the mesophilic (37^{o}C) and thermophilic (47^{o}C and 55^{o}C) acidogenic anaerobic bacteria to be compared. To maintain the required culture temperature to within ± 0.1^{o}C, each reactor was provided with a temperature controller set at the required temperature. The culture reactors were seeded with 1 L of anaerobic sludge obtained from the mesophilic anaerobic digester of a local municipal wastewater treatment plant (Woodman Point) and 1 L of synthetic complex wastewater of 4 g total COD/L. The cultures were continuously stirred using magnetic stirrers and semi-continuously drained and fed five times daily with synthetic complex wastewater (4 g total COD/L) at 4.8 hours interval to maintain a hydraulic retention time of one day. Any biogas produced was lost through the reactor's side-arm effluent outlet and biogas outlet at the top of the reactor.

Figure 3.1 shows the set-up of the three continuously-stirred insulated acidogenic culture reactors with their respective temperature controllers positioned at the left side of the reactors and their synthetic feedwaters and feeding pumps positioned at the rear of the reactors.

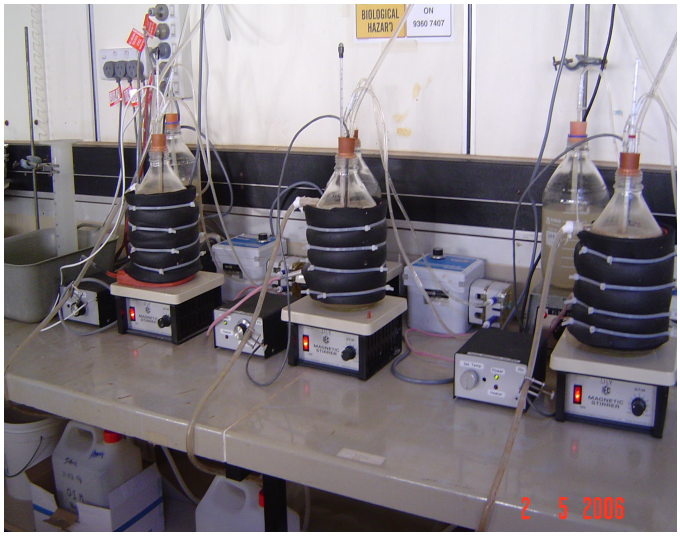

Figure 3.1. Insulated acidogenic culture reactors at 37°C (left), 47°C (middle) and 55°C (right)

3.2.2. Semi-continuous acid-phase anaerobic reactors

Two computer-controlled laboratory-scale anaerobic continuously stirred tank reactors (CSTRs), one made of plexiglass and the other of borosilicate glass, of total volume 1.2 L and working volume 0.9 L were used in parallel in all the semi-continuous acid-phase reactor experiments on the synthetic complex wastewater and raw piggery wastewater. For comparative thermophilic and mesophilic reactor experiments, one of the reactors was operated at thermophilic temperature of 55°C and the other reactor at mesophilic temperature of 37°C. Heating of the thermophilic (55°C) reactor content was by means of a thermostat-controlled built-in heater at the base of the plexiglass reactor while the mesophilic (37°C) reactor employed an immersion heating probe to heat the borosilicate glass reactor content. Overhead mechanical stirrers with speed of 180 rpm were used to mix the reactor contents while mixing of the feedwater was carried out using magnetic stirrer bars. Draining and feeding of the reactors were carried out by four Masterflex C/L Cole-Palmer peristaltic pumps which were connected to a desktop computer via a four-socket power supply block. All pump tubing for the feedwater, effluent and biogas was made of Masterflex tygon tube number 17. The two reactors were connected to a desktop computer which was installed with *National Instrument* Labview software. The software was programmed to control the feedwater and effluent pumping times as well as real-time data logging of the reactors' temperatures and biogas production.

At regular fixed time intervals, a known quantity of effluent was pumped out of the reactor followed by the same volume of chilled feedwater being pumped into the reactor in a semi-continuous mode to maintain the specified HRT. To prevent microbial degradation of the feedwater and effluent during the HRT runs, the bottles were stored in poly-foam containers containing icepacks that were replaced twice a day. Figure 3.2 shows the set-up of the two computer-controlled acid-phase reactors and their associated accessories

Figure 3.2. Experimental set-up of the two computer-controlled anaerobic acidogenic continuously stirred tank reactors (CSTRs)

In Figure 3.2, the thermophilic reactor was located next to the computer on the far left-hand side while the mesophilic reactor was located on the far right-hand side. Feedwaters (chilled in poly-foam containers and seated on top of the magnetic stirrers) and the peristaltic pumps (feed pumps stacked on top of the effluent pumps) of the thermophilic and mesophilic reactors were located at the right- and left-hand side of the reactors respectively. Figure 3.3 shows the schematic diagram of the reactors' set-up.

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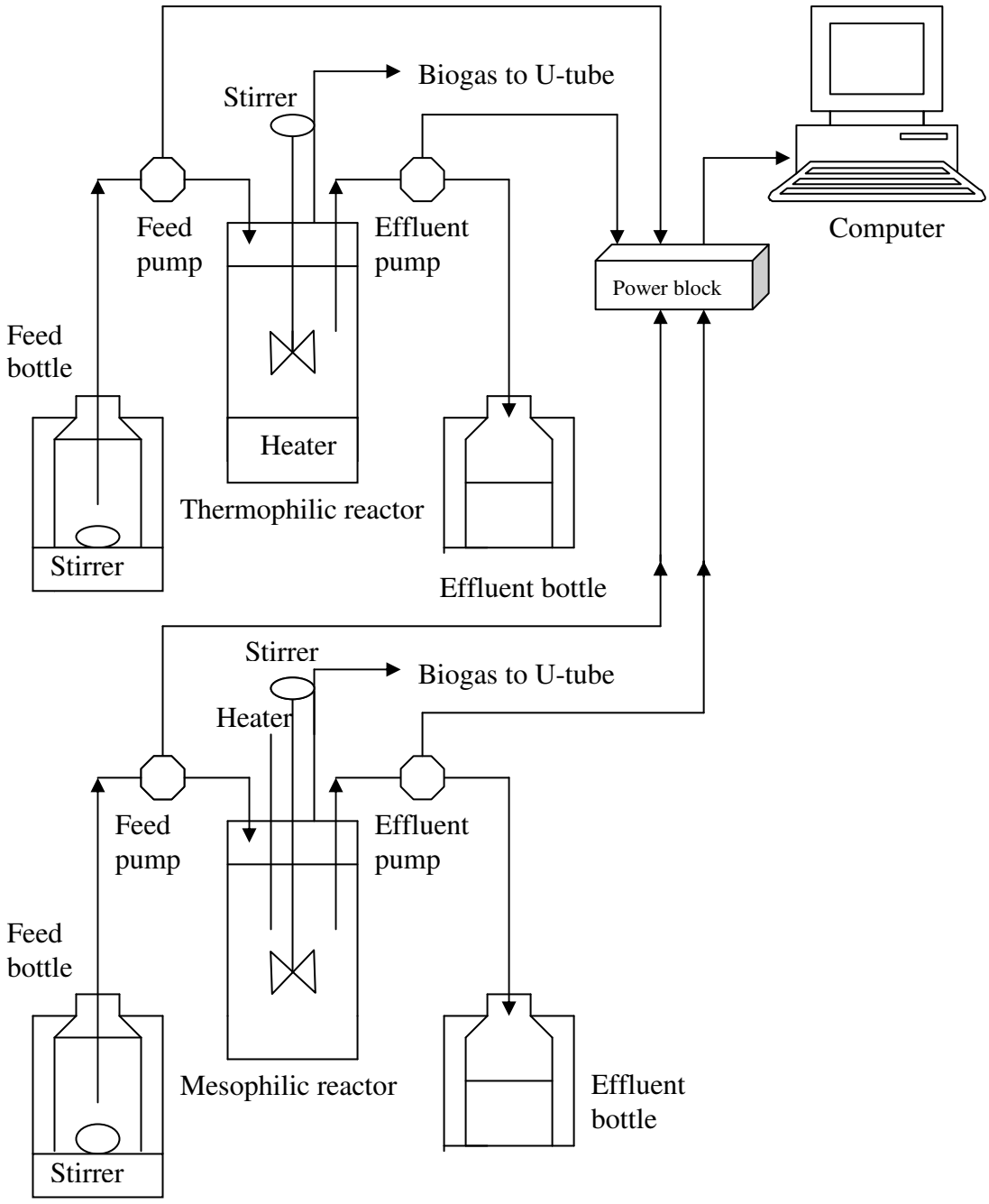


Figure 3.3. Schematic diagram of the automated anaerobic acid-phase reactors (CSTRs) set-up

3.2.3. Synthetic complex wastewater

As it is impossible to prepare synthetic wastewater that matches the physico-chemical characteristics of real complex piggery wastewater as used in the pilot-scale reactors at South Australia, a decision was made to use commercial weaner pig feed

pellets which contained a mixture of undissolved and dissolved carbohydrates, proteins, fats and other organic matter (lupins, dehulked lupins, wheat, triticale, groats, canolameal, soyameal, fishmeal, meatmeal, bloodmeal, molasses, tallow, salt, enzyme, lysine, methionine, threonine, choline, vitamin/trace mineral premix) as a compromise feed source for the preparation of synthetic complex organic wastewater in deionised water. Table 3.1 gives the nutrients data of the weaner feed pellets supplied by the manufacturer, Milne Feed Pty Ltd, Western Australia.

Table 3.1. Typical analysis for weaner feed pellets

Nutrients	As feed basis (dry)
Crude protein (min.)	20.0%
Added salt (max.)	0.15%
Available lysine, DE	0.75%
Calcium (min.)	0.90%
Available phosphorus (min.)	0.45%

The feed pellets were ground with a coffee grinder prior to use to facilitate dissolution and to minimise tubing blockage. As the commercial feed pellets already contained vitamins and trace minerals in its formulation, no further addition of these minerals was added to the synthetic wastewater. Similarly, no further additions of nitrogen and phosphorus were necessary as the synthetic wastewater had an estimated COD:N:P ratio of 40:3:1, which was considerably above the minimum nutrient requirement of 400:5:1 in terms of COD:N:P (Kasapgil *et al.*, 1995). Table 3.2 gives the key physico-chemical characteristics of the commercial pig feed as supplied by the manufacturer and the prepared synthetic complex wastewater for the culture reactors as measured by the test methods described in this chapter.

Table 3.2. Physico-chemical characteristics of commercial pig feed and synthetic complex wastewater for the culture reactors

Analysis	Commercial pig feed pellet (data from manufacturer)	Synthetic complex wastewater (for 5.23 g/L pig feed)
pH		6.2-6.4
Total COD (g/L)		3.85 ± 0.77
Soluble COD (g/L)		0.64 ± 0.11
Particulate COD (g/L)		3.21
Total Solids		0.28% (w/v)
Volatile Solids	94.7 % (w/w)	0.28 % (w/v)
Total Suspended Solids		0.23% (w/v)
Volatile Suspended Solids		0.23% (w/v)
Total Kjeldahl Nitrogen	29,000 mg-N/kg	351 mg-N/L (152 mg-N/5.23 g)
Nitrate & Nitrite (mg/kg)	1.4	
Total Phosphorus	6100 mg-P/kg	101 mg-P/L (32 mg-P/5.23 g)
Total Organic Carbon	37 % C	
Water Insoluble Matter	16.2 ± 0.9 % (w/w)	

Data of mean values of triplicate analysis (± standard deviation)

3.3. GENERAL ANALYTICAL METHODS

3.3.1. Mixed liquor analysis

3.3.1.1. pH

Sample pH was measured using a portable Hanna HI 8424 microprocessor pH meter.

3.3.1.2. Total alkalinity (APHA Standard Methods 2320 B)

Standard 0.1*N* HCl was regularly standardised against 0.05*N* Na_2CO_3 solution. A 20 mL mixed liquor sample was titrated promptly with the standardised 0.1*N* HCl (N) to pH 4.5 (T). Total alkalinity was calculated as $CaCO_3$ (mg/L) = T * N * 50000/sample (mL)

3.3.1.3. Total solids, volatile solids, total suspended solids and volatile suspended solids (APHA Standard Methods 2540 B)

Mixed liquor sample size of between 3 and 5 mL was used for the solids determination as per Standard Method 2540 B.

3.3.1.4. Ortho-phosphate (Lachat automated flow injection analyser QuickChem Method 31-115-01-3-A)

Selected mixed liquors and centrifuged samples were sent to a third party NATA accredited laboratory (Marine and Freshwater Research Laboratory (MAFRL) at Murdoch University) for ortho-phosphate analysis.

3.3.1.5. Sulphate (Lachat automated flow injection analyser QuickChem Method 10-116-10-1-C)

Selected mixed liquors and centrifuged samples were sent to a third party NATA accredited laboratory (Marine and Freshwater Research Laboratory (MAFRL) at Murdoch University) for sulphate analysis.

3.3.1.6. Chemical oxygen demand (APHA Standard Methods 5220 C closed reflux titration method)

For total COD determination, whole mixed liquor sample was used. For soluble COD determination, mixed liquor sample was centrifuged at 13,000g for 5 min and the clear liquor decanted for COD testing. A 5 mL diluted sample was treated with sulphuric acid reagent. Well-mixed sample was digested in a block digester preheated to 150^{o}C and refluxed for 2 hr inside the fume cupboard. A 0.05 mL ferroin indicator was added to the cooled sample and titrated with standardised 0.1*M* ferrous ammonium sulphate (FAS) to the end-point as shown by a colour change from blue-green to reddish-brown.

3.3.1.7. Ammonia-nitrogen (Hach Nessler method)

A 2 mL diluted centrifuged sample (13,000g for 5 min) was treated with 25 µL mineral stabiliser, 25 µL polyvinyl alcohol dispersing agent and 0.1 mL Nessler reagent. Sample was mixed well and allowed to react for 1 min. Sample absorbance was measured with a spectrophotometer at 425 nm wavelength and its ammonia-nitrogen concentration determined from the prepared 4-point standard ammonia-nitrogen calibration graph.

3.3.1.8. Volatile fatty acids (VFAs by Gas Chromatography method)

A 0.9 mL centrifuged sample (13,000g for 5 min) was acidified with 0.1 mL 10% formic acid prior to VFA analysis. A 1 µL of the acidified sample was analysed for acetate, propionate, i- and n-butyrate, i- and n-valerate, i- and n-caproate by Gas Chromatograph Varian Star 3400 Model equipped with EC-1000 mega-bore column of 15 m x 0.53 mm x 1.2 µm and a flame-ionisation detector. Temperatures of the column, injector and detector were 80^{o}C, 200^{o}C and 250^{o}C respectively. The VFA concentrations were determined from the prepared four-point standard mixed VFA calibration graphs.

3.3.1.9. Carbohydrates (Morris, 1948)

A 1 mL diluted sample was treated with 1 mL cold anthrone reagent solution (0.1%) at 100^{o}C and cooled at 4^{o}C for 10 min. Sample absorbance was measured with a spectrophotometer at 625 nm wavelength and its glucose concentration determined from the prepared 4-point standard glucose calibration graph.

3.3.1.10. Proteins (Lowry *et al*., 1951)

A 0.1 mL diluted sample was treated with 2 mL reagent mixture (2% Na_2CO_3, 1% sodium potassium tartarate and 0.5% $CuSO_4.5H_2O$) and incubated at 10 min at room temperature. Sample was then treated with 0.2 mL of diluted Folin-Phenol solution and incubated for 30 min. Sample absorbance was measured with a spectrophotometer at 600 nm wavelength and its protein concentration determined from the prepared 5-point standard bovine serum albumin (BSA) calibration graph.

3.3.1.11. Lipids (APHA Standard Methods 5520 B partition-gravimetric method)

A known quantity (10 mL or more, depending on oil content) of acidified mixed liquor was extracted with n-hexane three times and the clear organic layer from each extraction was combined. A known quantity (10 mL or more) of the organic fraction was evaporated at 85^{o}C, and then cooled in a dessicator before weighing to determine the oil in mg/L.

3.3.1.12. Fluorescence *in situ* hybridisation or FISH (Advanced Wastewater Management Centre, University of Queensland)

A known quantity of the mixed liquor (0.5 mL for synthetic wastewater and 0.25 mL for real piggery wastewater) was promptly fixed with 1 mL filtered para-formaldehyde (4%, pH 7.2) for 1 to 3 hr. Cells were pelletised by centrifuging at 5,000 g for 5 min and fixative decanted. Cells were then washed with phosphate buffer saline (130mM sodium phosphate buffer, 130mM NaCl, pH 7.2), centrifuged, decanted and cells resuspended in 1:1 volumes of the same phosphate buffer saline and 99.5% absolute ethanol. Cells were stored at -20^{o}C for later analysis.

Duplicate 4 µL of the well-mixed diluted sample were spotted on the wells of an 8-well Teflon-coated slide and let to air dry on the bench. After air drying, the slide was dehydrated in an ethanol series of 50%, 80% and 98% for 3 min each and then air dried. The dehydrated slide was then hybridised with the group-specific oligonucleotide probes. A 9 µL hybridisation buffer (0.9M NaCl, 0.01% SDS, 20mM Tris/HCl, pH 7.2) was added to each sample well on the slide, followed by 0.5 µL of the available group-specific oligonucleotide probes (50 ng/µL) as shown in Table 3.3.

Table 3.3. Group-specific oligonucleotide probes from ThermoHybaid, Germany

Probe name	Target group
ARC-915	*Archaea*
EUBMIX	Bacteria
Alpha-968	Alpha-Proteobacteria
Beta-42a	Beta-Proteobacteria
Gamma-42a	Gamma-Proteobacteria
LGC	Low GC bacteria
HGC	High GC bacteria

Slide was gently swirled to ensure sample mixture was well mixed. The slide was placed in a 50 mL polypropylene screw top falcon tubes containing a tissue moistened with hybridisation buffer. The closed tube was incubated in a moisture chamber at 46^{o}C for 1 to 2 hr. After hybridisation, the slide was carefully removed from the tube and rinsed immediately with wash buffer (5M NaCl, 1M Tris/HCl, 0.5M EDTA, 10% SDS + Milli-Q water) at 48^{o}C. Slide was then placed into the wash buffer tube and let to immerse for 10 to 15 min in the water bath at 48^{o}C. After the wash, the slide was rinsed and immersed in chilled water at 4^{o}C for 2 to 3 sec to remove the salt. The washed slide was immediately air dried using compressed air.

Sample wells in the dried slide were mounted in anti-bleaching agent (DABCO) and viewed under Olympus BX51 photomicroscope fitted with a mercury light source and equipped with blue and green filters and an attached Olympus DP70 digital camera. Eight to ten microscopic fields were randomly selected for digital images to be taken at x 40 magnification. The digital images were processed with Paint Shop Pro 5.01 software and the hybridised fluorescent cells per field were enumerated. Based on the initial sample volume, dilution factor and total fields per sample well, the average fluorescent cells per mL sample were calculated.

3.3.1.13. Real-time PCR (Skillman *et al*., 2009)

Primer pairs selected to quantify all bacteria and methanogens (Table 3.4) were included in 20 µL real-time PCR mixtures containing 10 µL SYBR Green mix (IQ SYBR Green Supermix; Bio-Rad), 7 µL distilled water, 1 µL forward primer (10µM), 1 µL reverse primer (10µM) and 1 µL DNA.

Real-time PCR amplification was conducted on a Rotorgene 3000 (Corbett Life Science, Sydney, Australia) according to the manufacturer's instruction. Amplification was initiated by denaturation at 95^{o}C for 10 min followed by up to 40 cycles of denaturation at 95^{o}C for 15 sec and annealing at 55^{o}C for bacteria and 50^{o}C for methanogens for 30 sec, and then by extension at 72^{o}C for 30 sec.

Table 3.4. PCR primers selected for quantification of microbial populations

Primer	Target group	Position in 16S	Sequence
Fq	16S gene, all bacteria and methanogens	1097	CGGCAACGAGCGCAACCC
Rq	16S gene, all bacteria and methanogens	1221	CCATTGTAGCACGTGTGTAGCC
MET630F	16S gene, methanogens	630	GGATTAGATACCCSGGTAGT
MET803R	16S gene, methanogens	803	GTTGARTCCAATTAAACCGCA
CperfF	16S gene, *Clostridium perfringens*	176	CGCATAACGTTGAAAGATGG
CperfR	16S gene, *Clostridium perfringens*	258	CCTTGGTAGGCCGTTACCC
Cperf Probe		190	5'-[FAM]TCATCATTCAACC AAAGGAGCAATCC[TAMRA]-3'

Fluorescence was acquired during extension using an excitation wavelength of 470 nm and emission detection at 530 nm. A final melting curve analysis was carried out by continuously monitoring fluorescence between 55^{o}C and 95^{o}C with 0.5^{o}C increments every 10 sec. Threshold cycles were calculated automatically by Rotorgene software (Version 6), standardized amounts of DNA extracted from enumerated cultures were included in each run to monitor and correct any between-run variability.

Fisher Biotech PCR reagents were used for Taqman real-time PCR for *C. perfringens.* Each 20 µL reaction contained 1µL hybridisation probe (2µM), 2 µL forward primer (10µM), 2 µL reverse primer, 2 µL PCR buffer, 2 µL dNTPs (2mM), 2 µL $MgCl_2$ (25mM), 0.2 µL Hotstart Taq, 7.8 µL dH_2O and 1 µL DNA. The cycling conditions were as follows: an initial 10 min step at 94^{o}C for Taq activation, followed by 35 cycles of denaturation at 94^{o}C for 10 sec, annealing at 55^{o}C for 20 sec, and extension at 70^{o}C for 10 sec. Fluorescence was acquired during the annealing step.

3.3.2. Biogas volume and composition (CH_4, CO_2, H_2 by Gas Chromatography method)

Biogas production was measured by a plexiglass U-tube fitted with a float switch on one end and partially filled with silicone oil. The U-tube was connected to the reactor, a 3-way solenoid valve and an electronic gas counter. The latter registered the volume of biogas produced per day as total counter clicks. The counter click was pre-calibrated with a known quantity of air that was dependent on the observed biogas production rate from the reactor (Chugh *et al*., 1998).

50 µL of the biogas produced was sampled at the gas outlet and analysed for methane, carbon dioxide and hydrogen using Gas Chromatograph Varian Star 3400 Model equipped with Porapak Q 80/100 packed column and thermal conductivity detector. High purity nitrogen was used as the column carrier gas and temperatures of the column, injector and detector were set at 40^{o}C, 120^{o}C and 120^{o}C respectively. Concentrations of methane, carbon dioxide and hydrogen were determined from their respective 4-point standard calibration graphs prepared from standard pure methane gas and standard gas mixture of 60% hydrogen and 40% carbon dioxide. Daily methane, carbon dioxide and hydrogen production rates were calculated from the volume of biogas produced and the methane, carbon dioxide and hydrogen concentrations in the biogas based on the following equation:

$V\text{-}CH_4$ or or $V\text{-}CO_2$ or $V\text{-}H_2$ = (V-b * Tc) * % CH_4 or % CO_2 or % H_2

Where: $V\text{-}CH_4$ = volume of methane produced

$V\text{-}CO_2$ = volume of carbon dioxide produced

$V\text{-}H_2$ = volume of hydrogen produced

V-b = volume of measured biogas

Tc = temperature correction

% CH_4 or % H_2 = measured methane or hydrogen concentration

The volume of biogas was corrected to standard temperature (0^{o}C).

CHAPTER 4
BATCH VIAL EXPERIMENTS ON ANAEROBIC ACID-PHASE DIGESTION OF SYNTHETIC COMPLEX WASTEWATER

4.1. INTRODUCTION

Acid-phase anaerobic digestion process was first developed in the seventies in United States for the treatment of sewage sludge (Pohland and Ghosh, 1971). It forms the first-phase or stage of the anaerobic digestion process that comprises the hydrolysis and acidogenesis steps in Figure 2.2 (Chapter 2). It is used as a pre-treatment system that precedes the anaerobic digester or the enhanced biological nutrient removal system, where insoluble organic macromolecules of carbohydrates, proteins and lipids are hydrolysed to soluble monomers of sugars, amino acids and long-chain fatty acids respectively. The soluble organics are further converted to volatile fatty acids, alcohols and other intermediate products by the acid-forming fermentative bacteria. The primary role of an acid-phase anaerobic digester is thus to maximise the hydrolysis of insoluble degradable organic matter and acidification of the soluble organic matter to VFAs for conversion to methane biofuel in the conventional anaerobic digestion system, or for used as soluble carbon and energy sources in the enhanced biological denitrification and phosphorus removal processes (Güngör *et al*., 2009; Elefsiniotis *et al*., 2004, 1993; Skalsky and Daigger, 1995; Randall *et al*., 1994).

This chapter presents experimental findings from two preliminary batch vial experiments on the acid-phase anaerobic digestion of synthetic complex wastewater prepared from commercial pig feed.

The first batch experiment was designed to address a research question on what effect would temperature at the region between the optimum mesophilic (37^{o}C) and thermophilic (55^{o}C) temperatures had on the anaerobic acid-phase bioconversion of organic matters. It is widely believed that the performance of fermentative bacteria in the conversion of organic substrates was sub-optimum at temperatures between the optimum mesophilic and thermophilic temperatures. However, scientific findings on

this issue were contradictory (Section 2.4.3. in Chapter 2). Therefore, the objective of the first batch vial experiment was to examine whether acidogenic anaerobic bacteria cultivated at the so-called sub-optimum temperature of 47^{o}C would exhibit the worst fermentation performance compared to at 37^{o}C (mesophilic optimum) and 55^{o}C (thermophilic optimum). It was hypothesised that cultivation of acidogenic anaerobic bacteria at the transition temperature of 47^{o}C would select fermentation bacteria capable of maximum conversion of organic substrate to fermentation products at the cultivated temperature (47^{o}C).

The second batch vial experiment was designed to investigate the effect of pH on the solubilisation and acidification of the organic matter present in the synthetic complex wastewater at mesophilic (37^{o}C) and thermophilic (55^{o}C) temperatures. With the synthetic complex wastewater used in this study containing a mixture of undissolved particulate organic materials and dissolved organic matter, this batch vial experiment was designed with the following objectives: 1) to examine the effects of acidic to alkaline pH on the solubilisation and acidification of particulate organic matter; and 2) to establish the optimum working pH for use in the next continuous acid-phase reactor experiments with this synthetic complex organic wastewater.

4.2. MATERIALS AND METHODS

4.2.1. Effect of temperature on the fermentation performance of acidogenic anaerobic bacteria

4.2.1.1. Objective

To investigate whether the conversion of wastewater organic carbon components to volatile fatty acids by the mixed anaerobic microorganisms cultivated at 47^{o}C (transition temperature) was greater than at 37^{o}C (mesophilic) or 55^{o}C (thermophilic).

4.2.1.2. Method

Figure 3.1 shows the acidogenic culture reactor at 47^{o}C. After three months of cultivation at 47^{o}C under acidogenic conditions, the culture was used to study the effect of temperature at 37^{o}C, 47^{o}C (control) and 55^{o}C on microbial solubilisation and acidification of the organic components in the culture. As the culture at 1-day

HRT still contained a significant amount of particulate COD at around 3 g/L, it was decided that no additional ground pig feed substrate would be added to the wastewater as originally planned to prevent potential substrate inhibition. Instead, total and soluble COD, volatile fatty acids, biogas volume and compositions were monitored over 5 days of incubation in 125 mL serum glass vials at 37^{o}C, 47^{o}C and 55^{o}C.

As pH 5.5 was reported to be optimum for acidogenesis of wastewater organics (Yu and Fang, 2002, 2003), the reactor culture at 47^{o}C was adjusted from its initial pH 4.0 to pH around 5.5 (pH 5.8) with 10M sodium hydroxide (NaOH) under nitrogen purging. Following this, the wastewater at time zero was sampled for COD (total and soluble) and VFA analysis. For each of the three temperatures under study, triplicate vials containing 90 mL wastewater for mixed liquor monitoring and triplicate vials containing 60 mL for biogas monitoring were prepared under nitrogen purging.

Prior to capping the vials, the headspace was again purged with nitrogen to displace the air. Prepared vials were placed in shaking water baths set at 37^{o}C, 47^{o}C and 55^{o}C. After 20-min of incubation, the headspace of all sample vials were degassed using plastic syringe-needle to commence the 5-day incubation experiment. A sample of 10 ml mixed liquor was withdrawn daily after 24-hr elapsed using plastic syringe attached with a sterile 18-gauge needle from each of the vial set aside for COD (total and soluble) and VFA analysis. During sampling, the vials were inverted and the contents shaken to ensure representative mixed liquors were withdrawn for analysis. For biogas compositional analysis, a 100 µL gas-tight glass syringe-needle was used to sample 20 to 50 µL of headspace gas daily from the sample vials set aside for gas monitoring. Concurrently, daily biogas production was measured using water displacement by inserting a 22-gauge fine needle with attached tubing to the sample vials. At the end of the 5 days incubation, all sample vials were opened for final pH analysis.

4.2.2. Effect of pH on solubilisation and acidification of organic matter

4.2.2.1 Objectives

The objectives of this experiment were: 1) to examine the effects of acidic to alkaline pH on the solubilisation and acidification of particulate organic matter at mesophilic (37^oC) and thermophilic (55^oC) temperatures; and 2) to establish the optimum working pH for use in the next continuous acid-phase reactor experiments with this synthetic complex organic wastewater.

4.2.2.2. Method

Figure 3.1 shows the acidogenic culture reactors at 37^oC and 55^oC. After about six months of cultivation under acidogenic conditions at 1-day HRT, the cultures at 37^oC and 55^oC were used to study the effect of pH at their respective temperatures on microbial solubilisation and acidification of the organic components in the cultures. The initial reactor wastewater pH at 37^oC and 55^oC were adjusted from their initial pH of 3.8 and 4.9 respectively to pH of 6, 7 and 8 with 10M NaOH under nitrogen purging. Triplicate control and test samples were prepared by transferring 25 mL of the wastewater into 38 mL serum glass vials under nitrogen purging. To overcome the sampling difficulties encountered in previous experiment, modification to sample vial preparation was introduced in this experiment. Here, separate sets of sample vials were prepared so that one set could be sacrificed for daily chemical analysis during the four-day incubation period. Prior to capping the vials, the headspace was again purged with nitrogen to displace the air. Prepared vials were then placed in their respective water baths at 37^oC and 55^oC. The same procedures of incubation, sampling and analysis as described in section 4.2.1 were adopted with the exception that one set of sample vials was taken daily for pH, COD (total and soluble) and VFA analysis.

4.3. RESULTS

4.3.1. Effect of temperature on the acidogenic anaerobic bacteria cultivated at 47°C (batch experiment 1 as described in section 4.2.1)

Table 4.1 gives the total COD, total VFA-COD (acetate, propionate, n- & i-butyrate, n- & i-valerate) and hydrogen-COD data at 37°C, 47°C and 55°C.

Table 4.1. Total COD, total VFA-COD and hydrogen-COD concentrations at 37°C, 47°C and 55°C

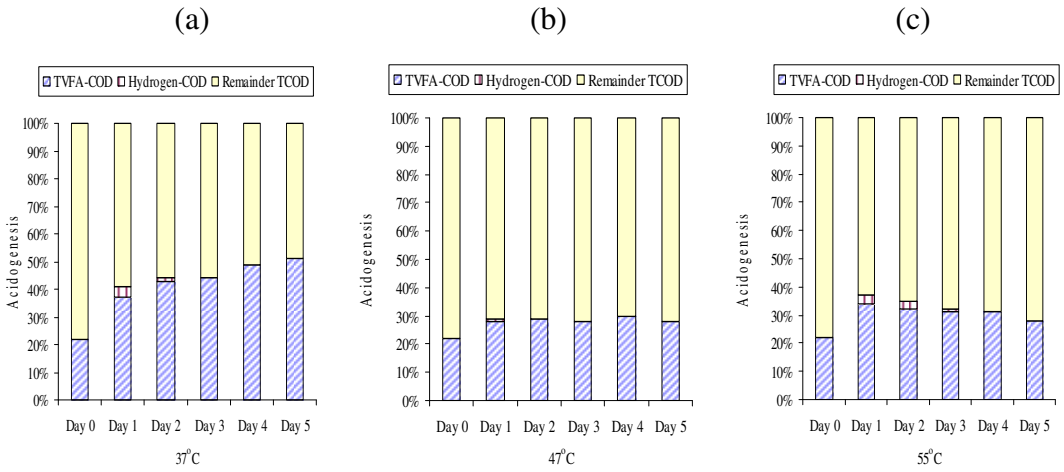
Incubation day	Temperature (°C)	Total COD (mg/L)	Total VFA (mg COD/L)	Hydrogen (mg COD/L/d at 0°C)
0	1-d HRT culture at 47°C	5910 (350)	1307 (124)	0
1	37	3883 (65)	2166 (135)	253 (8)
	47	3995 (233)	1682 (80)	74 (1)
	55	4256 (404)	2024 (125)	177 (15)
2	37	3323 (517)	2552 (21)	64 (14)
	47	4069 (323)	1706 (28)	0
	55	4443 (65)	1876 (26)	168 (9)
3	37	3323 (233)	2619 (63)	0
	47	3659 (171)	1646 (46)	0
	55	3883 (505)	1851 (26)	54 (17)
4	37	4779 (65)	2867 (85)	15 (3)
	47	4181 (323)	1788 (41)	0
	55	3248 (0)	1821 (33)	28 (17)
5	37	5600 (0)	3003 (291)	3 (5)
	47	4313 (296)	1668 (57)	1 (2)
	55	3976 (65)	1653 (118)	20 (19)

Data are mean values of triplicate measurements (± standard deviation)

As the initial total COD in the mixed liquor is conserved in an acid-phase anaerobic system through its conversion to intermediate products such as VFA, other metabolites and reduced gases such as hydrogen and/or methane (Yu *et al*., 2002a; Eastman and Ferguson, 1981), the consistently lower total COD concentrations by as much as 40% in some samples between day 1 and day 5 relative to the initial sample at day 0 suggested that the sampling method using syringes with restrictive needle apertures was inadequate for sampling heterogeneous mixed liquor that contained undissolved particles.

Despite the shortcoming in the sampling method, the COD material balance of the cultures at 37°C, 47°C and 55°C (expressed as mg COD/L of the initial total COD in the 47°C culture) illustrated in Figures 4.1(a), (b) and (c) respectively clearly highlight that at the three test temperatures of 37°C, 47°C and 55°C, the easily

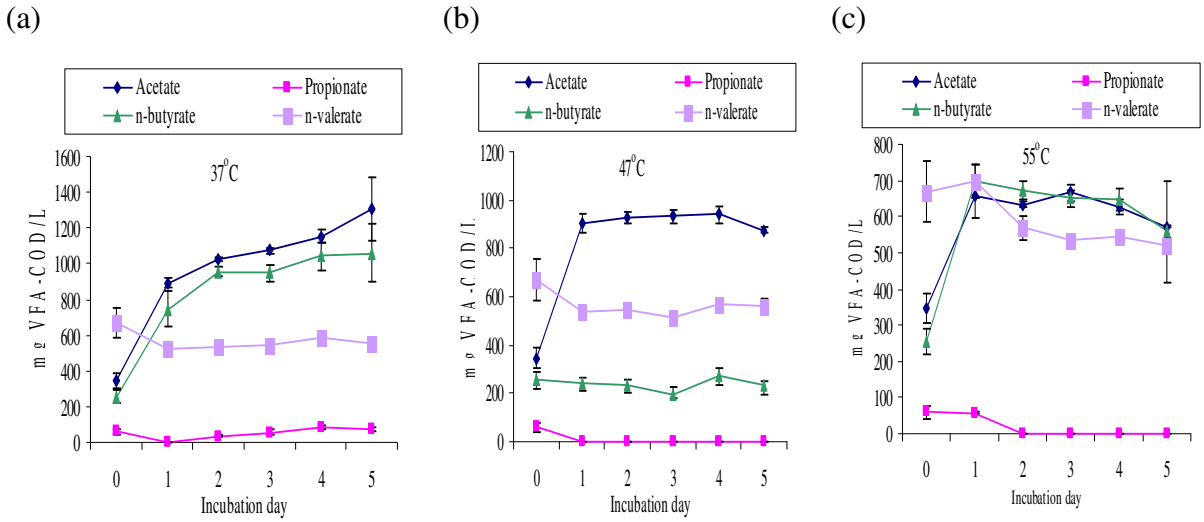
degradable organic matter were hydrolysed and acidified to VFAs during the first day of incubation.



Figures 4.1 (a), (b) and (c). Profiles of COD material balance at 37°C, 47°C and 55°C as a function of incubation period

This was evidenced by the statistically significant increase in soluble COD in the form of VFAs and hydrogen gas. The increase in acidified products (total VFA and hydrogen) was observed to be highest at mesophilic temperature of 37°C compared to thermophilic temperatures of 47°C and 55°C which were comparable. The small amount of biogas produced at the three temperatures contained hydrogen plus carbon dioxide and no detectable methane. Given that the reduced hydrogen gas has a low COD value of 0.714 g/L H_2 (Eastman and Ferguson, 1981), its COD reduction contribution was therefore negligible. The lack of methane in the biogas indicated that the methanogens had been effectively inhibited in the acidic environment. The material balance profiles at the three test temperatures indicate that more than half of the initial complex substrate remained undigested after 5-day of incubation.

Figures 4.2 (a), (b) and (c) show the VFA production trends at 37°C, 47°C and 55°C relative to the initial culture (control).



Figures 4.2 (a), (b) and (c). Volatile fatty acid production trends at 37°C, 47°C and 55°C as a function of incubation period (error bars indicate standard deviations)

The trends clearly show significant increases in acetate production rates at 37°C, 47°C and 55°C over the initial culture during the first day of incubation. However, the acetate increase was lowest at 55°C (90 ± 12%) compared to 37°C (159 ± 9%) and 47°C (162 ± 12%). While acetate continued to show slight increase with time at mesophilic temperature of 37°C, there were no further increases occurring at thermophilic temperatures of 47°C and 55°C. The profiles also showed significant increases in n-butyrate production at 37°C (194 ± 38%) and 55°C (175 ± 18%) during the first day of incubation. However, there was no similar increase being observed at 47°C. There were also no further increases in propionate and n-valerate productions at the three test temperatures. By the end of the 5-day incubation, the culture pH had dropped from their initial pH of 5.8 to pH 4.0 at 37°C, pH 4.5 at 47°C and pH 4.3 at 55°C respectively as a result of the increase in volatile fatty acids production.

4.3.2. Effect of pH on solubilisation and acidification of organic matter (batch experiment 2 in section 4.2.2)

4.3.2.1. Batch vial acid-phase treatment at 37°C

Table 4.2 gives the pH (initial and final), COD (total and soluble), total VFA-COD (acetate, propionate, n- & i-butyrate, n- & i-valerate) and hydrogen-COD

concentrations at 37^{o}C. With the improved experimental design, the percentages total COD of the test samples relative to the initial samples were considerably improved to within ± 10% variations. The latter were mainly due to the inherent variable nature of the culture that contained undissolved particles which made sampling representative samples a challenging task.

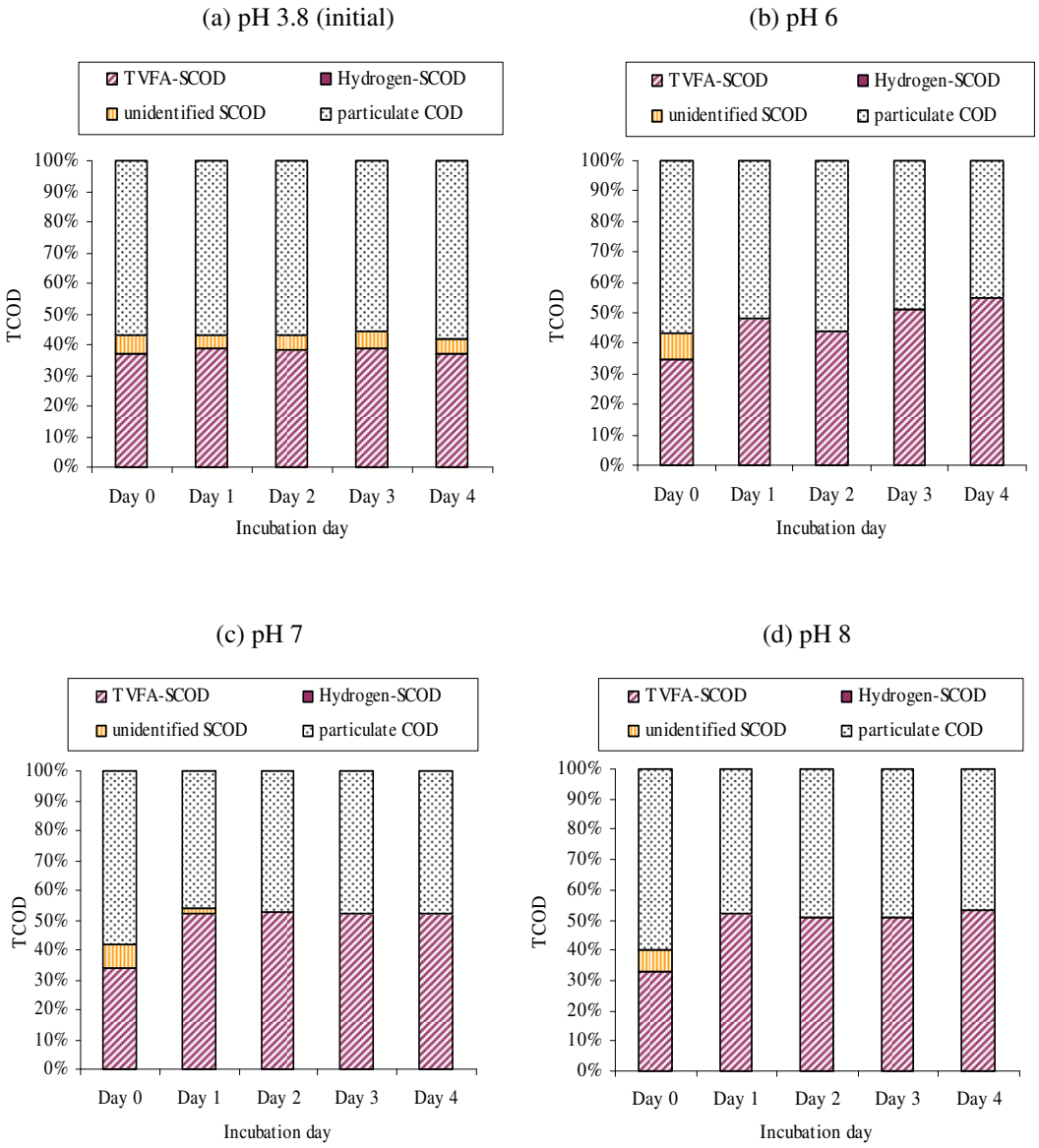
Similar to the COD material balance in the previous experiment, the acidified products measured in mg/L VFAs and hydrogen gas production at 37^{o}C are expressed as mg COD per litre of their respective initial total COD at day 0. Figures 4.3 (a), (b), (c) and (d) illustrate the COD material balance at pH 3.8 (initial), pH 6, pH 7 and pH 8 respectively as a function of incubation day.

Some 10% improvements in organics solubilisation were apparent when the mesophilic culture pH was raised from 3.8 to 6, 7 and 8. Slightly higher solubilisation was obtained at pH 7 and 8 than at the acidic pH of 3.8 and 6. The material balance profiles also highlight that the additional organics solubilisation and acidification had occurred during the first day of incubation and there were no further significant improvements thereafter. These observations supported the earlier observation of the previous vial experiment with the 47^{o}C culture in regards to organics solubilisation. Minute amount of biogas which consisted of hydrogen plus carbon dioxide and no detectable methane was produced at the adjusted pH of 6, 7 and 8. The COD contribution from hydrogen was negligible.

Table 4.2. pH (initial and final), COD (total and soluble), total VFA and hydrogen gas production rate at 37°C

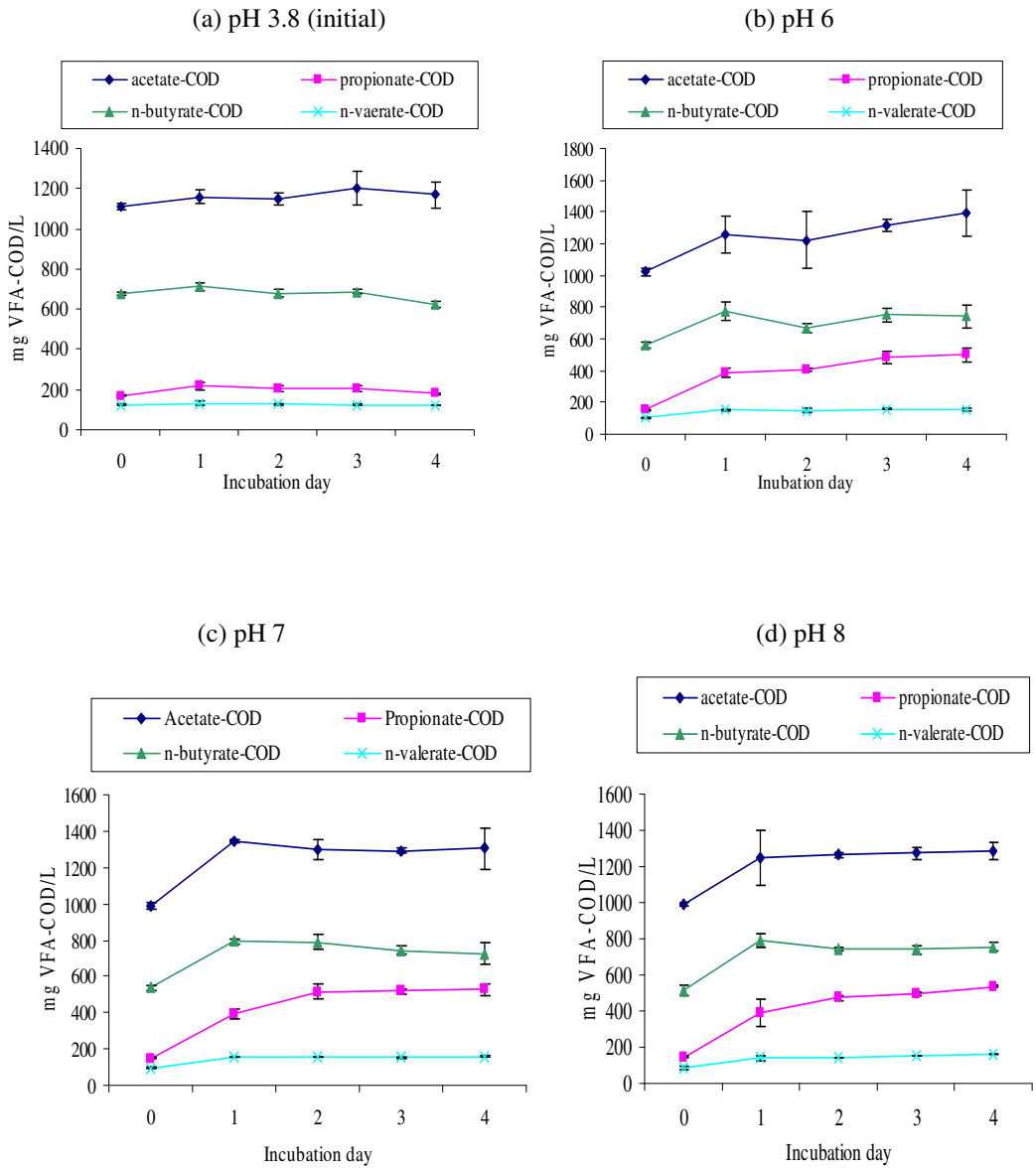
Incubation day	Initial pH	Final pH	Total COD	Soluble COD	Total VFA	Hydrogen production
			mg/L	mg/L	mg COD/L	mg COD/L/d
0	3.8	3.8	5677 (174)	2443 (49)	2077 (25)	0
0	6	6	5325 (122)	2264 (48)	1839 (49)	0
0	7.1	7.1	5198 (110)	2203 (49)	1771 (28)	0
0	8	8	5182 (73)	2059 (66)	1735 (30)	0
1	3.8	3.7	5560 (230)	2458 (13)	2223 (70)	0
1	6	5.8 (0.0)	5107 (96)	2491 (159)	2569 (202)	10 (2)
1	7.1	6.3 (0.0)	5198 (137)	2798 (57)	2684 (52)	1 (1)
1	8	6.5 (0.0)	5773 (179)	2385 (278)	2701 (112)	4 (1)
2	3.8	3.7	5503 (160)	2443 (45)	2161 (50)	0
2	6	5.8 (0.0)	4966 (252)	2443 (340)	2335 (70)	0
2	7.1	6.3 (0.0)	5746 (379)	2844 (260)	2758 (139)	2 (1)
2	8	6.4 (0.0)	5620 (190)	2912 (143)	2619 (29)	0
3	3.8	3.7 (0.0)	5488 (176)	2481(100)	2215 (102)	0
3	6	5.9 (0.2)	4989 (154)	2484 (29)	2707 (177)	0
3	7.1	6.2 (0.0)	4971 (343)	2541 (152	2710 (49)	1(0)
3	8	6.1 (0.2)	5335 (383)	2688 (375)	2663 (50)	6 (0)
4	3.8	3.8	5395 (173)	2362 (63)	2093 (79)	0
4	5.9	5.6 (0.0)	5208 (368)	2257 (69)	2794 (278)	0
4	7	6.2 (0.0)	5244 (178)	2330 (207)	2715 (209)	0
4	8	6.3 (0.0)	5630 (96)	2394 (0)	2737 (73)	5 (0)

Data are mean values of triplicate measurements (± standard deviation)



Figures 4.3 (a), (b), (c) and (d). Profiles of COD material balance at pH 3.8 (initial), pH 6, pH 7 and pH 8 respectively as a function of incubation day at 37^{o}C

Figures 4.4 (a), (b), (c) and (d) show the effects of pH on VFA production trends at the initial pH 3.8, pH 6, 7 and 8 at 37°C relative to their controls at day 0 which contained acetate, propionate, n-butyrate and n-valerate.



Figures 4.4 (a), (b), (c) and (d). Volatile fatty acid production trends at pH 3.8 (initial), pH 6, pH 7 and pH 8 respectively as a function of incubation day (error bars indicate standard deviations)

The VFA production trends clearly show significant increases in acetate, propionate, n-butyrate and n-valerate productions at pH 6, 7 and 8 during the first day of incubation. The increases in VFA production caused the wastewater final pH to drop

(Table 4.2). While acetate, n-butyrate and n-valerate showed no further increases thereafter, propionate continued to increase slightly with time.

4.3.2.2. Batch vial acid-phase treatment at 55°C

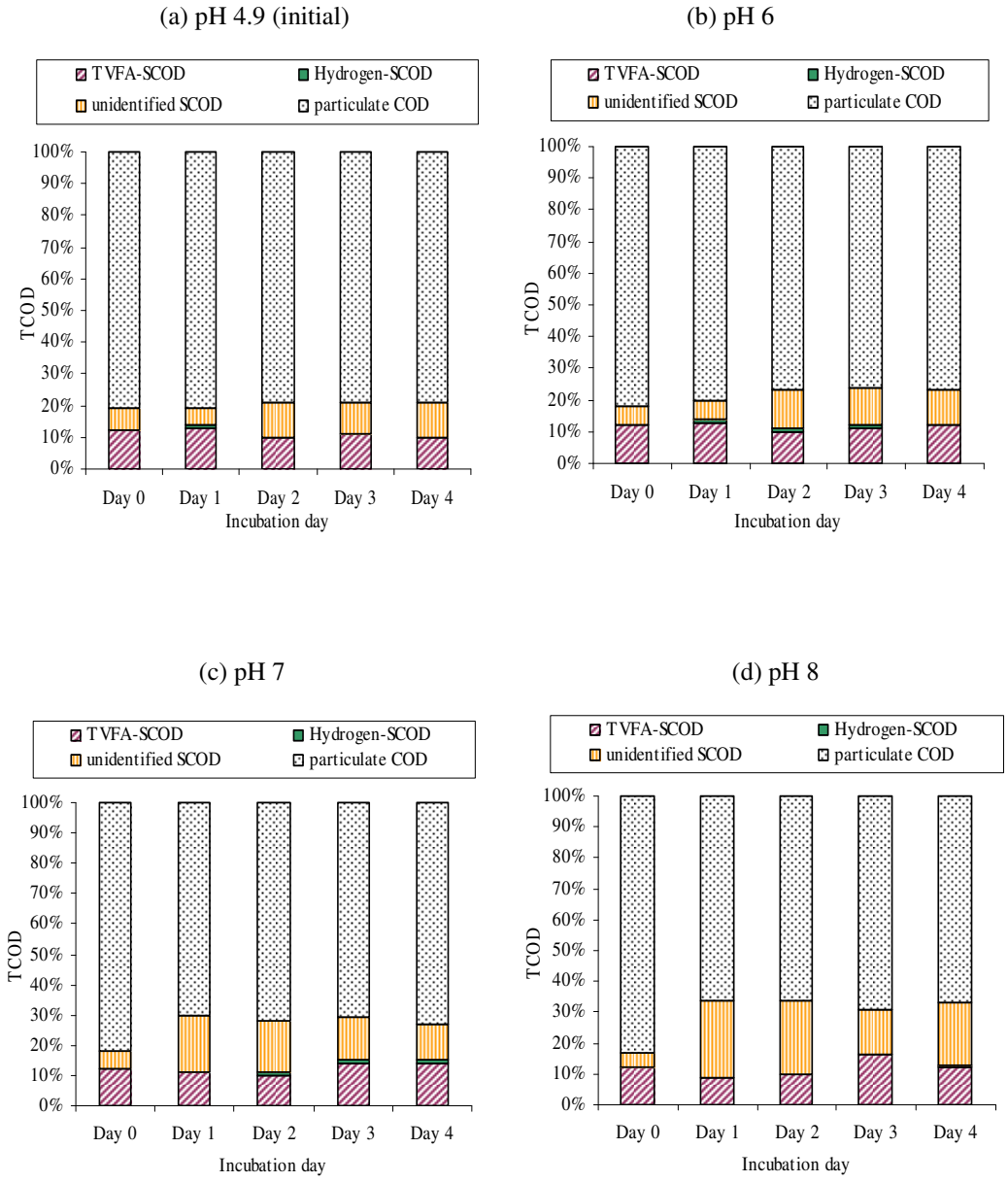
Table 4.3 gives the pH (initial and final), COD (total and soluble), total VFA-COD (acetate, propionate, n- & i-butyrate, n- & i-valerate) and hydrogen-COD concentrations at 55°C.

Table 4.3. pH (initial and final), COD (total and soluble), total VFA-COD and hydrogen-COD concentrations at 55°C

Incubation day	Initial pH	Final pH	Total COD	Soluble COD	Total VFA	Hydrogen
			mg/L	mg/L	mg COD/L	mg COD/L/d
0	4.9	4.9	5387 (430)	1004 (0)	664 (7)	0
0	5.9	5.9	5363 (292)	983 (26)	641 (14)	0
0	7	7	5107 (339)	908 (19)	622 (22)	0
0	8	8	5069 (277)	875 (31)	606 (39)	0
1	4.9	4.8	5263 (299)	1028 (6)	678 (14)	32 (1)
1	5.9	5.5 (0.0)	5803 (211)	1091 (21)	678 (9)	42 (6)
1	7	6.7 (0.1)	5580 (105)	1518 (116)	548 (32)	22 (3)
1	8	7.2 (0.0)	5803 (211)	1711 (171)	480 (5)	11 (1)
2	4.9	4.9	5397 (380)	1119 (22)	521 (88)	0
2	5.9	5.4 (0.0)	5555 (171)	1252 (94)	550 (20)	31 (10)
2	7	6.4 (0.1)	6180 (260)	1446 (105)	495 58)	40 (20)
2	8	6.7 (0.1)	5476 (302)	1716 (142)	495 (17)	25 (20)
3	4.9	4.8	5462 (245)	1125 (22)	567 (60)	0
3	5.9	5.2 (0.1)	5774 (42)	1290 (43)	596 (21)	29 (20)
3	7	6 (0.3)	6106 (217)	1500 (88)	707 (84)	45 (30)
3	8	6.1 (0.5)	6279 (516)	1587 (138)	792 (79)	18 (10)
4	4.9	4.8	5540 (160)	1107 (13)	548 (34)	0
4	5.9	5 (0.1)	6220 (42)	1213 (39)	653 (34)	16 (20)
4	7	5.8 (0.2)	6012 (421)	1394 (78)	734 (96)	43 (20)
4	8	6.6 (0.0)	6299 (286)	1667 (68)	605 (57)	31 (10)

Data are mean values of triplicate measurements (± standard deviation)

Figures 4.5 (a), (b), (c) and (d) illustrate the COD material balance at pH 4.9 (initial), pH 6, pH 7 and pH 8 respectively as a function of incubation day.



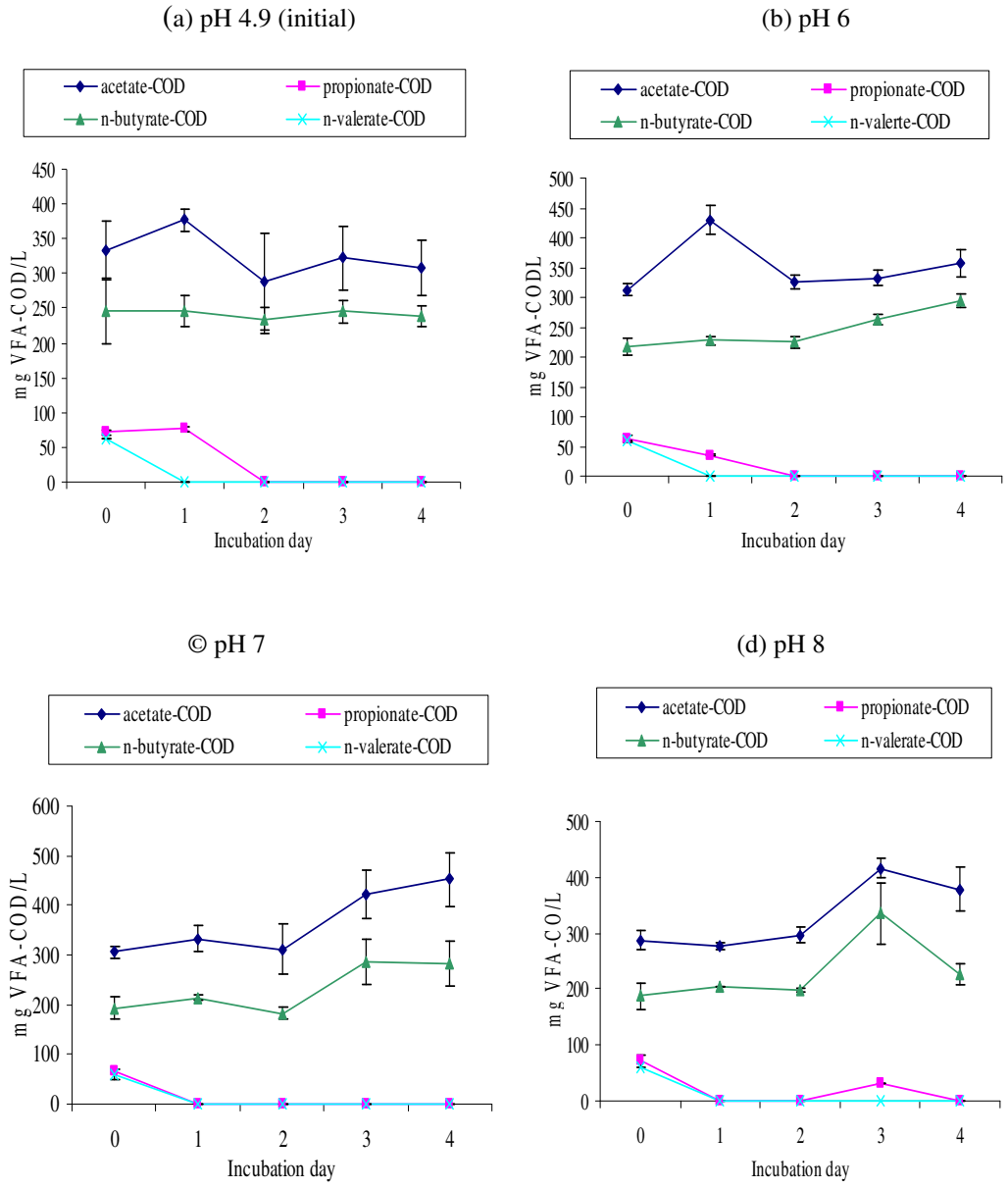
Figures 4.5 (a), (b), (c) and (d). Profiles of COD material balance at pH 4.9 (initial), pH 6, pH 7 and pH 8 respectively as a function of incubation day at 55^{o}C

As observed at 37^{o}C with pH elevation, improvements in organics solubilisation were apparent when the thermophilic culture pH was raised from 4.9 to 6, 7 and 8. The material balance profiles also highlight that the additional organics solubilisation occurred during the first day of incubation and there were no further significant improvements thereafter. While solubilisation was significantly higher at neutral pH 7 and alkaline pH 8 than at the acidic pH of 4.9 and 6, a lag period of 2 days was

observed before the soluble organics were acidified. As a result of the increased VFA concentrations, the final pH of the adjusted samples dropped from their initial pH 6, 7 and 8 to pH 5, 6 and 7 respectively at the end of the 4-day incubation period.

Figures 4.6 (a), (b), (c) and (d) show the effects of pH on VFA production trends at the initial pH 4.9, pH 6, 7 and 8 at 55^{o}C relative to their controls at day 0 which contained acetate, propionate, n-butyrate and n-valerate. The VFA trends show significant increase in acetate production at pH 6 during the first day of incubation while increases in acetate and n-butyrate productions at pH 7 and pH 8 only occurred during the third day of incubation. Small amount of biogas which consisted of hydrogen plus carbon dioxide and no detectable methane was produced by all the samples at 55^{o}C.

Raising the wastewater pH to 6 was observed to stimulate the hydrogen production on day 1 while raising the pH further to 7 and 8 resulted in an initial lag of 1 day for pH 7 and 2 days for pH 8 before hydrogen production was stimulated compared to the control at pH 4.9. The observed large gas production variations from day 2 onwards were likely to be due to leaky rubber stoppers caused by daily puncturing of the rubber stoppers for biogas measurements.



Figures 4.6 (a), (b), (c) and (d). Volatile fatty acid production trends at pH4.9 (initial), pH 6, pH 7 and pH 8 respectively as a function of incubation day (error bars indicate standard deviations)

4.3.2.3. Comparison of batch acid-phase anaerobic treatment at 37°C and 55°C

For similar feed concentration of about 5 g total COD/L at 1-day HRT, soluble COD and total VFA concentrations of the mesophilic culture at 37°C (Table 4.2) were significantly greater than the thermophilic culture at 55°C (Table 4.3) by two-fold. However, significantly higher hydrogen production of five- to ten-fold and greater amount of non-VFA soluble organics was observed at 55°C than at 37°C. At both

mesophilic and thermophilic temperatures, more complex organics were hydrolysed and acidified to soluble form at pH 7 and 8 than at the acidic pH of 4, 5 and 6. However, at thermophilic temperature of 55^{o}C, acidification of the soluble organics at pH 7 and 8 occurred only after a lag period of 2 days which reflected the length of time it took the biomass to adapt to the new pH environment. The latter observation suggested that thermophilic microorganisms were more sensitive to environmental changes in short-term vial experiment than their mesophilic counterparts.

4.4. DISCUSSION

4.4.1. Effect of temperatures on the acidogenic anaerobic bacteria cultivated at 47^{o}C

The observed increase in soluble COD (inclusive of hydrogen) and VFAs at 37^{o}C and 55^{o}C over the control at 47^{o}C after one day of incubation (Table 4.1) suggested that the anaerobic biomass cultivated at 47^{o}C was made up of a mixture of mesophilic and thermophilic microbial populations. These observations were somewhat similar to the observations of Bouskova *et al.* (2005) and Chen (1983) that there existed a small percentage of thermophiles in the mesophilic sludge which quickly dominated under thermophilic anaerobic conditions. However, in this study, the mesophiles which were originally present in the thermophilic wastewater at 47^{o}C quickly dominated over the thermophiles when the wastewater temperature was lowered from 47^{o}C to 37^{o}C. The conversion performance of the mesophilic acidogenic bacteria clearly out-performed their thermophilic counterparts in the acidification of dissolved organic matter.

The significantly lower total VFA concentrations at thermophilic temperatures of 47^{o}C and 55^{o}C compared to mesophilic temperature of 37^{o}C could not have been attributed to methanogenic degradation of VFA as the biogas did not contain detectable methane and the effluent pH of 4 to 4.5 was far too acidic for the methanogens. It is postulated that there were either fewer number of thermophilic fermentation bacteria present to acidify the organic substrate or the thermophilic microorganisms were subject to greater product inhibition under low pH and higher temperature conditions. At low pH and high temperature, VFAs exist as un-ionised or protonated forms which are more toxic than the ionised forms as they diffuse more

rapidly through the cell membrane of microorganisms, causing their internal pH to drop and disrupting their metabolic functions (Geraldi, 2006). The higher acidification at 37^{o}C compared to at 55^{o}C obtained in this study was in agreement with Pavan *et al.* (2000) who reported considerably higher total VFAs concentration in the pilot-scale mesophilic (35^{o}C) hydrolytic anaerobic reactor than the thermophilic (55^{o}C) reactor treating the organic fraction of municipal solid waste.

The observation that hydrolysis and acidification at 47^{o}C were comparable to those at 55^{o}C did not support the hypothesis that cultivation of acidogenic biomass at the transition temperature between mesophilic temperature of 37^{o}C and thermophilic temperature of 55^{o}C would select microorganisms with maximum organics conversion capability. The observation suggested that the activity of anaerobic microorganisms cultivated at 47^{o}C were not adversely affected by temperature elevation to 55^{o}C. This finding differed from Zoetemeyer *et al.* (1982a) who observed a significant drop in the acidogenesis performance of acid-phase CSTR on glucose substrate between the optimum mesophilic and thermophilic temperature ranges of 36-38^{o}C and 51-53^{o}C respectively as well as higher acidogenesis at thermophilic optimum temperature. This finding also differed from Fang and Yu (2001) and Yu and Fang (2003) who reported slight increase in acidogenesis of lactose and gelatine respectively in UASB acid-phase reactors at temperatures between 20^{o}C and 55^{o}C. The differences in findings between these studies could be due to the wastewater composition and reactor configuration used which are known to influence the type of microbial populations selected and subsequently the substrate conversion kinetics (sections 2.3 and 2.4 in Chapter 2).

Although microbial data were not available from all these studies, the organic substrate used in this study was complex and consisted of insoluble particulate and soluble organic carbohydrates, proteins and lipids as opposed to the simple soluble glucose (monosaccharide), lactose (disaccharide) and gelatine (protein) used in the studies of Zoetemeyer *et al.* (1982a), Fang and Yu (2001) and Yu and Fang (2003) respectively. In addition, the reactors used in Fang and Yu (2001), Yu and Fang (2003)'s studies were upflow anaerobic sludge blanket (UASB) configuration which is capable of retaining large amount of biomass (section 2.3 in Chapter 2) as opposed to the suspended-growth CSTR used in this study and Zoetemeyer *et al.* (1982a).

4.4.2. Effect of pH on solubilisation and acidification of organic matter at thermophilic and mesophilic temperature

The results from this study demonstrated that pH-adjustment of the acidic wastewater to neutral-alkaline pH of 7-8 improved solubilisation and acidification of the organic component in the complex organic substrate. These results concurred with Zhang *et al.* (2005) and Penaud *et al.* (1997). Zhang *et al.* (2005) found pH 7 to enhance solubilisation and acidification of real kitchen waste compared to the adjusted pH 5, 9 and 11. Penaud *et al.* (1997) reported increased COD solubilisation and VFA production from the protein component of complex real pharmaceutical wastewater with increasing pH from 5 to 9. As the final pH of the pH-adjusted cultures (pH 7 and 8) remained at 6 to 7, no pH control was deemed necessary for the next semi-continuous reactor experiments.

4.5. CONCLUSIONS

The results from the first batch vial study clearly did not support the hypothesis that acidogenic fermentative bacteria cultivated at the transition temperature between mesophilic and thermophilic optimum temperatures would select and enrich microorganisms with maximum fermentative performance at the cultivated temperature over other temperatures. Hydrolysis and acidification of the complex organics was observed to be highest at 37^{o}C (mesophilic) than at 47^{o}C and 55^{o}C (thermophilic) which showed comparable performance. The organics conversion results indicate that the microbial populations cultivated at the transition temperature of 47^{o}C were comprised of mixed populations of mesophilic and thermophilic acidogenic anaerobic bacteria. Upon shifting the culture's temperature from 47^{o}C (low thermophilic) to 37^{o}C (mesophilic) and 55^{o}C (thermophilic), the growth of mesophilic and thermophilic microbial populations were stimulated and became dominant at their respective preferred environmental temperature. The implications from these findings were that acid-phase anaerobic digestion could be performed at the lower thermophilic temperature of 47^{o}C instead of the conventional thermophilic temperature of 55^{o}C without compromising the hydrolysis and acidification of the organic carbon compounds; and the acid-phase anaerobic digestion could be

performed more cost-effectively at mesophilic temperature of 37^{o}C than at thermophilic temperatures of either 47^{o}C or 55^{o}C.

The results of the second batch vial study indicated that for this synthetic wastewater, prior adjustment of its initial acidic pH of 6 to pH between 7 and 8 would be beneficial in improving the solubilisation and acidification of the organic carbon compounds during subsequent anaerobic digestion.

CHAPTER 5
SEMI-CONTINUOUS REACTOR EXPERIMENTS ON ANAEROBIC ACID-PHASE DIGESTION OF SYNTHETIC COMPLEX WASTEWATER

5.1. INTRODUCTION

Hydraulic retention time (HRT) is one of the many process parameters that affect the microbial performance of the hydrolysis-acidification anaerobic reactor as it determines the achievable extent of hydrolysis of particulate organic matter and acidification of the soluble organic components (Guerrero *et al*., 1999; Banerjee *et al*., 1998; Alexiou *et al*., 1994). Therefore, it is important that the optimum HRT for effective treatment of a particular wastewater type be established. As the acidogenic fermentative bacteria have very short generation time of about 30 minutes (section 2.1.2), very short HRT of between 6 and 24 hours has been recommended for the first acid-phase reactor of the two-phase anaerobic system to wash out the slow-growing syntrophic consortia of VFA-consuming acetogenic bacteria and methane-producing methanogens (Lettinga and Hulshoff Pol, 1986). However, it is unclear whether such short HRT would be effective for effective hydrolysis and acidogenesis of complex organic wastewaters that are commonly encountered in the livestock and food processing industries which contained undissolved and dissolved organic matter. For particulate-containing sludge, HRT of 2 to 3 days were reported to be necessary to produce maximum VFA (Puchajda and Oleszkiewicz, 2006) while the limited literature on two-phase (stage) anaerobic treatment of cattle and pig manure wastewaters showed longer HRT of 2 to 5 days were commonly applied to the acid-phase anaerobic reactors (Demirer and Chen, 2005; Harikishan and Sung, 2003; Cseh *et al*., 1984).

Temperature is another operating parameter that affects the performance of the anaerobic acid-phase reactor. While acid-phase anaerobic reactor operating at thermophilic temperature of 55^{o}C was more effective in the destruction of selected pathogenic organisms than at mesophilic temperature (Mitsdörffer *et al*. 1990; Lee *et al*., 1989), the findings on its solubilisation and acidification performance of organic

carbon compounds has often been contradictory. Guerrero *et al*. (1999) and Yu *et al*. (2002a) reported that thermophilic acid-phase reactors at 55^{o}C solubilised and acidified more organic carbon compounds than mesophilic acid-phase reactors at 37^{o}C. In contrast, Pavan *et al.* (2000) and Penaud *et al.* (1997) found mesophilic reactor at 35-37^{o}C to solubilise and acidify more organics than thermophilic reactor at 55^{o}C. Differences in substrate compositions and types of fermentative bacteria present could have contributed to the contradictory findings.

The objective of this experiment was to investigate and compare the effect of hydraulic retention times ranging between 1 and 4 days on the performance of thermophilic and mesophilic anaerobic acid-phase CSTRs in the conversion of complex organic wastewater to acidified products.

This chapter presents the experimental findings from the semi-continuous reactor experiments on the acid-phase anaerobic digestion of synthetic complex wastewater at thermophilic (55^{o}C) and mesophilic (37^{o}C) conditions.

5.2. MATERIALS AND METHODS

5.2.1. Objective

To assess and compare the effect of hydraulic retention times ranging between 1 and 4 days on the fermentation performance of thermophilic (55^{o}C) and mesophilic (37^{o}C) anaerobic acid-phase CSTRs in the conversion of complex organic wastewater to acidified products.

5.2.2. Method

Figures 3.2 and 3.3 (Chapter 3) show the photo and schematic diagram of the two automated semi-continuously pumped, continuously stirred thermophilic (55^{o}C) and mesophilic (37^{o}C) anaerobic acid-phase reactors set-up. Both reactors had a total volume of 1.2 L and were seeded to a working volume of 0.9 L with their respective anaerobic synthetic wastewaters from the acidogenic culture reactors that had been in operation for a year at 1-day HRT. The synthetic complex wastewater was prepared using ground weaner pig feed pellets and distilled water to give 4 g total COD/L concentration. Based on the findings of earlier preliminary batch experiment on pH

effect (section 4.2.2), the wastewater pH was adjusted from 6.0 to 8.0 with 10M sodium hydroxide to increase the soluble organics concentration. To prevent microbial degradation of the substrate, the feed bottles were placed in polystyrene-foam containers which contained ice packs that were replaced twice a day. Similarly, effluent bottles were kept in chilled poly-foam containers. The chilling method adopted was found to be adequate as shown by the absence of VFAs in the residual feed influent.

During the entire acidogenic digestion, pH of the reactors was intentionally left uncontrolled to assess the extent of solubilisation and acidification achievable with a low cost, simple to operate technology that could be used in the field. The anaerobic acid-phase reactors were operated from 4-day down to 1-day HRT on a semi-continuous mode of draining and feeding. The reactors were operated up to some 3 weeks minimum which was equivalent to five HRTs minimum for each HRT before assumed steady state effluent samples were taken for a suite of chemical and microbial analysis for eight consecutive days. Triplicate samples per reactor effluent per day were taken and analysed for chemical (total and soluble COD, ammonium-nitrogen, soluble phosphorus, solids as TS, VS, TSS, VSS, pH) and microbiological analysis (whole cell fluorescence *in situ* hybridisation or FISH). Two biogas samples per reactor per day were sampled for H_2, CO_2, CH_4 analysis.

The reactor performance or efficiency was evaluated based on the extent of net hydrolysis and acidification calculated as follows:

*(1) Extent of net hydrolysis (%) = (effluent SCOD – influent SCOD + H_2-COD[1] and/or CH_4-COD[1] lost)/influent TCOD * 100* (adapted from El-Mashad *et al*., 2004)

*(2) Extent of net acidification (%) = (effluent TVFA-COD[1] – influent TVFA-COD[1] + H_2-COD[1] and/or CH_4-COD[1] lost)/influent TCOD * 100*

[1] calculated from theoretical COD equivalents (Eastman and Ferguson, 1981)

The mean data were statistically compared using Excel data analysis software of Student's t-test. Statistical significance of the differences in means was determined at 95% confidence level.

5.3. RESULTS

pH, ammonium-nitrogen, soluble phosphorus

Table 5.1 gives the pH, ammonium-nitrogen and soluble phosphorus concentrations in the synthetic complex wastewaters and reactor effluents at 4- to 1-day HRT.

Table 5.1. pH, ammonium-nitrogen and soluble phosphorus concentrations of the synthetic wastewater influents and reactor effluents at 1- to 4-d HRT

Synthetic wastewater	HRT (d)	pH	NH_4^+-N (mg/L)	Soluble phosphorus (mg/L)
Influent to T-reactor	1	8.2 (0.2)	1.2 (0.1)	10.7 (2.8)
T-effluent	1	4.4 (0.1)	4.9 (0.8)	23.3 (0.7)
Influent to T-reactor	2	8.0 (0.1)	1.3 (0.0)	9.6 (0.5)
T-effluent	2	4.4 (0.0)	10 (1)	23.5 (0.5)
Influent to T-reactor	3	8.0 (0.0)	1.3 (0.1)	10.0 (0.0)
T-effluent	3	4.4 (0.1)	11.9 (2.2)	22.6 (0.5)
Influent to T-reactor	4	7.9 (0.0)	1.6 (0.1)	8.7 (1.3)
T-effluent	4	4.3 (0.1)	8.0 (4.3)	25.1 (3.3)
Influent to M-reactor	1	8.2 (0.1)	1.3 (0.1)	11.8 (0.4)
M-effluent	1	4.0 (0.1)	0.2 (0.0)	21.5 (1.3)
Influent to M-reactor	2	8.0 (0.1)	1.3 (0.0)	9.1 (0.4)
M-effluent	2	3.9 (0.1)	0.4 (0.2)	21.1 (1.5)
Influent to M-reactor	3	8.0 (0.1)	1.3 (0.1)	9.2 (0.4)
M-effluent	3	3.9 (0.2)	0.4 (0.2)	18.8 (1.0)
Influent to M-reactor	4	7.9 (0.0)	1.6 (0.1)	8.7 (1.3)
M-effluent	4	4.0 (0.1)	0.4 (0.2)	25.9 (5.2)

Data are mean values (± standard deviation) T (thermophilic) M (mesophilic)

It was observed that following anaerobic treatment in the first-stage thermophilic and mesophilic reactors, the influent pH dropped substantially from 8 to 4.4 and 3.9 respectively which coincided with the increase in soluble COD and volatile fatty acids production (Table 5.4). Ammonium-nitrogen and soluble phosphorus concentrations of the thermophilic effluents were higher than the influents by 10- and 2-fold resepectively, with 2-day being the optimum HRT for nutrient released from the proteinacous organic materials. In contrast, there were no increases in ammonium-nitrogen concentrations in the mesophilic effluent while the soluble phosphorus concentrations were elevated by 2-fold.

Solids (total, volatile, total suspended, volatile suspended)

Table 5.2 gives the total, volatile, suspended and volatile suspended solids concentrations in the influents and effluents of the thermophilic and mesophilic first-stage reactors. The high percentage influent VS/TS and VSS/TSS values of over 94% indicated that the dissolved and suspended solids predominantly consisted of organic matter with only minor proportion as inorganic matter.

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Table 5.2. Total and volatile suspended solids in the synthetic influents and effluents at 1- to 4-d HRT

Synthetic wastewater	HRT (d)	TS (g/L)	VS (g/L)	TSS (g/L)	VSS (g/L)	% VS/TS	% VSS/TSS
Influent to T-reactor	1	3.9 (0.4)	3.7 (0.4)	3.1 (0.5)	3.1 (0.4)	94 (2)	98 (4)
T-effluent	1	3.6 (0.2)	3.4 (0.2)	3.0 (0.2)	3.0 (0.2)		
Influent to T-reactor	2	4.5 (0.4)	4.2 (0.4)	3.6 (0.4)	3.4 (0.4)	95 (1)	95 (2)
T-effluent	2	3.6 (0.2)	3.3 (0.2)	3.1 (0.2)	3.0 (0.2)		
Influent to T-reactor	3	4.6 (0.0)	4.4 (0.1)	3.5 (1.4)	3.4 (1.2)	95 (1)	99 (4)
T-effluent	3	3.3 (0.2)	3.0 (0.1)	2.6 (0.4)	2.5 (0.3)		
Influent to T-reactor	4	3.4 (0.0)	3.2 (0.0)	2.7 (0.1)	2.6 (0.1)	94 (3)	97 (1)
T-effluent	4	na	na	na	na		
Influent to M-reactor	1	4.0 (0.7)	3.8 (0.7)	3.4 (0.5)	3.3 (0.3)	95 (2)	98 (2)
M-effluent	1	3.0 (0.2)	2.8 (0.2)	2.5 (0.2)	2.5 (0.2)		
Influent to M-reactor	2	4.0 (1.0)	3.7 (1.0)	3.5 (0.6)	3.4 (0.5)	94 (1)	96 (1)
M-effluent	2	2.6 (0.3)	2.4 (0.3)	2.2 (0.3)	2.1 (0.3)		
Influent to M-reactor	3	4.8 (0.0)	4.6 (0.1)	4.3 (0.2)	4.3 (0.0)	95 (3)	97 (1)
M-effluent	3	2.4 (0.1)	2.1 (0.1)	1.9 (0.3)	1.7 (0.2)		
Influent to M-reactor	4	3.4 (0.0)	3.2 (0.0)	2.7 (0.1)	2.6 (0.1)	94 (4)	97 (1)
M-effluent	4	2.0 (0.3)	1.7 (0.2)	1.6 (0.4)	1.4 (0.2)		

Data are mean values (± standard deviation) T (thermophilic) M (mesophilic) na (not available)

As shown in Table 5.3, large fluctuations in the solids removal data were observed which made the data comparisons statistically insignificant ($p<0.05$). A combination of sampling and measurement inconsistencies of the particulate-containing mixed liquor rather than reactor instability were likely causes that affected the accuracy of the solids measurement.

Table 5.3. Solids removal data at 1- to 4-day HRT

Reactor	HRT (d)	TS removal (%)	VS removal (%)	TSS removal (%)	VSS removal (%)
T	1	10 (9)	10 (9)	6 (9)	5 (8)
	2	18 (8)	21 (9)	12 (9)	11 (10)
	3	28 (4)	32 (3)	15 (19)	16 (18)
	4	Na	na	na	na
M	1	23 (13)	24 (14)	21 (15)	19 (15)
	2	32 (12)	35 (13)	38 (11)	38 (11)
	3	51 (2)	54 (3)	58 (7)	60 (5)
	4	40 (12)	47 (5)	40 (13)	45 (83)

Data are mean values (± standard deviation) T (thermophilic) M (mesophilic) na (not available)

In general, greater mean reductions in TS and VS than TSS and VSS were observed because of the inclusion of dissolved solids (soluble organic compounds) in TS and VS measurements. As one of the primary aims of the first-stage anaerobic reactor was to maximise organics solubilisation (hydrolysis), VSS removal data is considered a more approriate non-specific parameter for gauging the solubilisation efficiency of the first-stage anaerobic reactor. The mean VSS removal data of the thermophilic reactor were generally greater at 2- and 3-day HRT than at 1-day HRT while the VSS removal data of the mesophilic reactor was highest ($p<0.05$) at 3-day HRT. The overall VSS removal data clearly show that mesophilic treatment hydrolysed substantially more organics than thermophilic treatment.

COD, volatile fatty acids and biogas

Table 5.4 gives the COD (total and soluble) and total VFA-COD data of the synthetic influents and effluents of the thermophilic and mesophilic acidogenic reactors. The percentage ratio of soluble COD/total COD was calculated to determine the amount of organics that had solubilised in the prepared wastewater. Similarly, the percentage ratio of total VFA-COD/soluble COD was calculated to determine the amount of soluble organics that had acidified to volatile fatty acids in the synthetic influent.

Table 5.4. Chemical oxygen demand (total and soluble) and total VFA concentrations of the synthetic influents and reactor effluents at 1- to 4-d HRT

Synthetic wastewater	HRT (d)	Total COD (mg/L)	Soluble COD (mg/L)	Total VFA (mg COD/L)	SCOD/TCOD	TVFA/SCOD
Influent to T-reactor	1	4133 (920)	752 (29)	0	19 (3)	0
T-effluent	1	4813 (390)	1107 (40)	925 (89)		
Influent to T-reactor	2	3611 (123)	750 (24)	0	21 (1)	0
T-effluent	2	4889 (393)	1145 (54)	874 (91)		
Influent to T-reactor	3	4830 (635)	779 (86)	0	16 (1)	0
T-effluent	3	4762 (319)	1282 (60)	759 (94)		
Influent to T-reactor	4	3717 (275)	914 (74)	0	26 (5)	0
T-effluent	4	4442 (400)	1105 (102)	607 (46)		
Influent to M-reactor	1	4838 (527)	738 (46)	0	15 (1)	0
M-effluent	1	4382 (513)	1496 (146)	1680 (155)		
Influent to M-reactor	2	4450 (170)	744 (77)	0	17 (2)	0
M-effluent	2	4430 (303)	1790 (88)	2023 (219)		
Influent to M-reactor	3	4769 (670)	765 (30)	0	18 (2)	0
M-effluent	3	4002 (294)	1549 (69)	1423 (54)		
Influent to M-reactor	4	3717 (275)	914 (74)	0	26 (5)	0
M-effluent	4	3574 (287)	1737 (131)	1674 (188)		

Data are mean values (± standard deviation) T (thermophilic) M (mesophilic)

The low influent ratios of SCOD:TCOD highlighted that the majority of the total organic matter in the synthetic complex wastewater was in insoluble form. Despite inhomogeneity observed in the different batches prepared, they were found to consist primarily of carbohydrates and proteins with lipids being the minor organic component (Table 5.5). Substantial increases in the effluent soluble COD and total VFA were evident following thermophilic and mesophilic treatment in the anaerobic acidogonic reactor. The overall COD data show mesophilic treatment produced significantly greater amounts of soluble COD and total VFA than thermophilic treatment.

Wide differences were noted in the influent and effluent TCOD data in a few cases which contradicted with the COD conservation principle of the acid-phase anaerobic system that influent total COD is conserved as fermentation intermediate products in the reactors. The differences were largely the result of sampling inconsistency associated with heterogenous particulate-containing wastewater which distorted the analysis somewhat.

Table 5.5. Organic compounds in the synthetic wastewaters fed to the reactors

Synthetic wastewater	HRT (d)	Total carbohydrates (mg/L)	Soluble sugar (mg/L)	Total proteins (mg/L)	Soluble proteins (g/L)	Lipids (mg/L)
Influent to T-reactor	1	1251 (155)	268 (20)	1965 (26)	22 (2)	165 (35)
Influent to T-reactor	2	2669 (120)	194 (12)	1584 (15)	18 (1)	185 (28)
Influent to T-reactor	3	3191 (255)	281 (32)	1286 (30)	23 (4)	180 (23)
Influent to T-reactor	4	2624 (152)	168 (15)	2234 (120)	23 (3)	130 (25)
Influent to M-reactor	1	1829 (130)	226 (32)	1316 (32)	22 (3)	200 (25)
Influent to M-reactor	2	1736 (214)	270 (22)	1292 (25)	27 (4)	180 (12)
Influent to M-reactor	3	1890 (260)	269 (23)	1078 (130)	19 (8)	165 (33)
Influent to M-reactor	4	2624 (152)	168 (15)	2234 (120)	23 (3)	130 (25)

Data are mean values (± standard deviation) T (thermophilic) M (mesophilic)

Table 5.6 shows the thermophilic effluent VFAs to consist of n-butyrate (31-42%) as the major VFA component, followed by acetate (15-20%) at the four HRTs tested. Propionate (1-7%), n-valerate (0-7%) and caproate (1%) formed the minor acidified products while i-butyrate and i-valerate were negligible. No alcohols were detected.

In contrast, the mesophilic effluent VFAs consisted mainly of acetate (19-36%), propionate (10-17%), n-butyrate (19-29%), n-valerate (10-25%) and caproate (9-17%). No alcohols were detected. Higher concentrations of propionate, n-valerate and caproate were observed compared to the thermophilic effluent.

Table 5.6. Compositions of the volatile fatty acids in the influents and reactor effluents

Synthetic wastewater	HRT (d)	Acetate (mg COD/L)	Propionate (mg COD/L)	i-butyrate (mg COD/L)	n-butyrate (mg COD/L)	i-valerate (mg COD/L)	n-valerate (mg COD/L)	Caproate (mg COD/L)
Influent to T-reactor	1	0	0	0	0	0	0	0
T-effluent	1	244 (24)	78 (12)	nd	504 (59)	nd	87 (10)	12 (20)
Influent to T-reactor	2	0	0	0	0	0	0	0
T-effluent	2	238 (18)	80 (10)	nd	470 (50)	nd	80 (9)	nd
Influent to T-reactor	3	0	0	0	0	0	0	0
T-effluent	3	212 (27)	92 (12)	nd	439 (51)	nd	16 (7)	nd
Influent to T-reactor	4	0	0	0	0	0	0	0
T-effluent	4	206 (16)	7 (12)	nd	394 (34)	nd	nd	nd
Influent to M-reactor	1	0	0	0	0	0	0	0
M-effluent	1	432 (28)	246 (24)	13 (4)	464 (48)	nd	348 (42)	177 (29)
Influent to M-reactor	2	0	0	0	0	0	0	0
M-effluent	2	357 (40)	200 (28)	48 (5)	551 (69)	55 (3)	489 (70)	323 (45)
Influent to M-reactor	3	0	0	0	0	0	0	0
M-effluent	3	552 (14)	258 (23)	nd	301 (21)	nd	162 (18)	150 (5)
Influent to M-reactor	4	0	0	0	0	0	0	0
M-effluent	4	551 (72)	238 (31)	nd	432 (57)	14 (2)	286 (35)	156 (18)

Data are mean values (± standard deviation) nd (not detected)
T (thermophilic) M (mesophilic)

Thermophilic treatment of the synthetic wastewater produced small amount of biogas which was found to contain 34 to 54% hydrogen and 22 to 35% carbon dioxide at 1- to 4-day HRT (Table 5.7). The balance was assumed to be nitrogen which could not be measured as high purity nitrogen gas was used as the column elution gas.

Table 5.7. Biogas composition and hydrogen yields of the thermophilic (T) and mesophilic (M) acid-phase reactors

Reactor	HRT (d)	% H_2	% CO_2	H_2 yield (m^3/kg influent TCOD fed) corrected to 0^oC	% H_2-COD of TCOD fed corrected to 0^oC
T	1	54 (3)	31 (3)	0.025 (0.01)	1.8 (0.7)
	2	49 (3)	32 (4)	0.041 (0.014)	2.9 (1.0)
	3	47 (4)	35 (3)	0.029 (0.007)	2.1 (0.5)
	4	37 (5)	22 (3)	0.006 (0.004)	0.5 (0.3)
M	1	31 (6)	31 (2)	0.006 (0.002)	0.4 (0.1)
	2	12 (5)	38 (4)	0.002 (0.002)	0.1 (0.2)
	3	1 (1)	34 (4)	<0.001	<0.1
	4	2 (3)	33 (5)	<0.001	<0.1

Data are mean values of 8 measurements (± standard deviation) T (thermophilic) M (mesophilic)

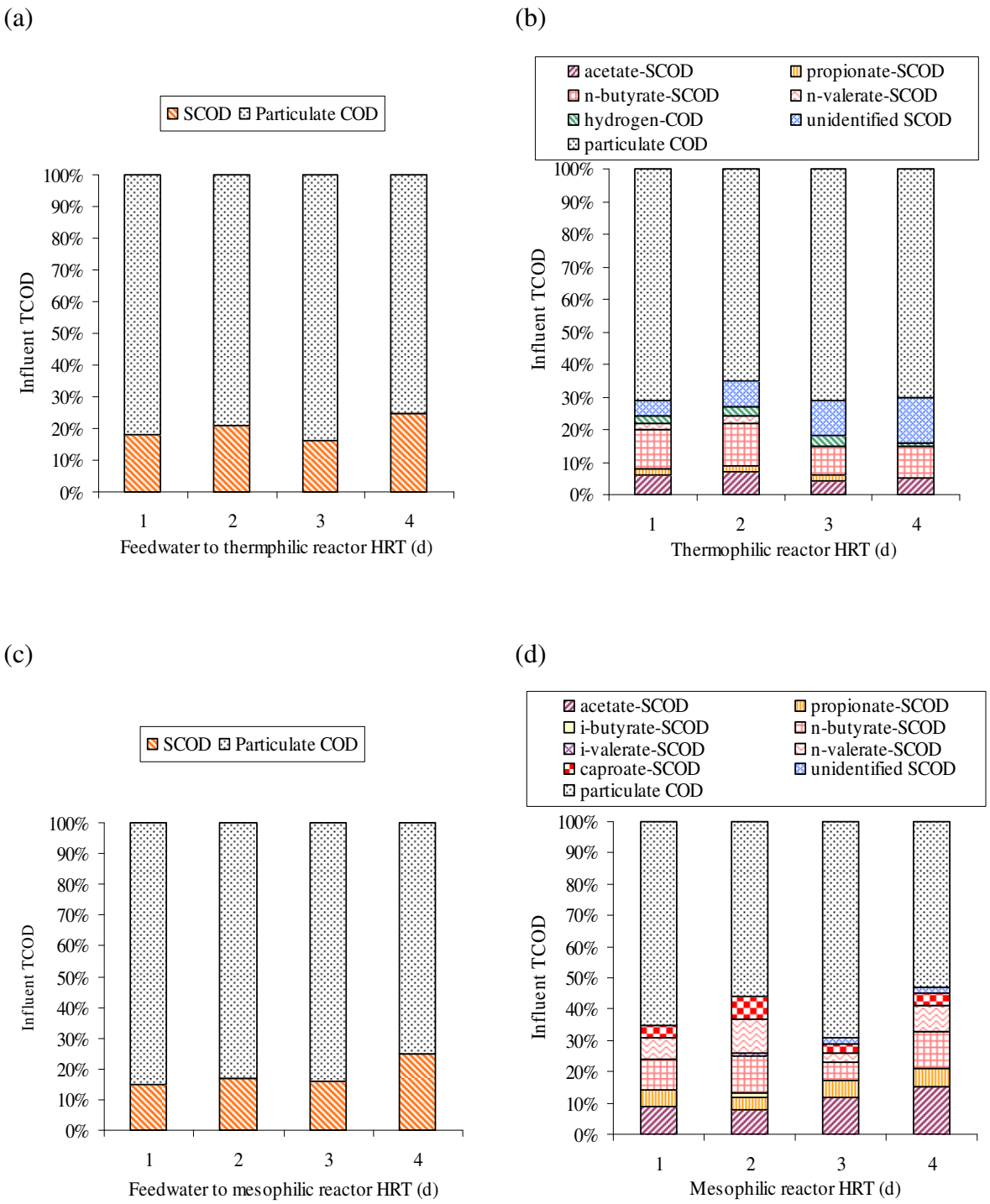
The lack of methane in the biogas was a clear indication that the methanogens which were confirmed present in the reactor effluent by whole cell *fluorescence in-situ hydrbidisation* (FISH) molecular method (Figure 5.4 (a)) had been effectively inhibited in the acidic effluent of pH 4.3. Statistical analysis ($p<0.05$) of the hydrogen yields indicates 2-day HRT had the highest yield.

In contrast, mesophilic treatment produced much lesser biogas compared to the thermophilic treatment. The biogas contained 1 to 31% hydrogen and 31 to 38% carbon dioxide at 1- to 4-day HRT, with hydrogen concentration increasing with decreasing HRT. Highest hydrogen yield, albeit very minute was obtained at 1-day HRT. The lack of methane again indicated that the methanogens present in the reactor effluent had been effectively inhibited in the acidic effluent of pH 3.9.

COD material balances

Figures 5.1 (a), (b), (c) and (d) show the COD material balances of the synthetic wastewater influents and effluents plus biogas from the thermophilic and mesophilic reactors constructed from the SCOD, VFA-COD and hydrogen-COD components relative to their respective influent TCOD concentrations. Hydrogen-COD (mg COD/L) was obtained by first converting the daily hydrogen production (L hydrogen/L/d) to mg hydrogen-COD/L/d using a conversion factor of 714 mg/L hydrogen (Eastman and Ferguson, 1981) and multiplying it by the HRT (d) (Hanaki *et al*., 1987). Figures 5.3 (a) and (b) displays the COD distribution of the various fermentation products in the soluble COD fractions of the thermophilic and mesophilic effluents respectvely.

Comparisons of the COD material balances of the influents fed to the reactors with the reactor effluents plus biogas shows that between 6 and 14% of the influent particulate organics were hydrolysed in the thermophilic reactor at 1- to 4-day HRT. This was manifested as increased effluent SCOD fractions (Figures 5.1 (a) and (b)). In the case of the mesophilic reactor, between 16 and 24% of the influent particulate organics were hydrolysed (Figures 5.1 (c) and (d)).



Figures 5.1 (a), (b). (c) and (d). COD material balance of the feedwaters and reactor effluents plus biogas of the thermophilic and mesophilic reactors as a function of hydraulic retention time

Figure 5.1 (b) shows the soluble organic matter of the thermophilic reactor effluent was made up predominantly of VFA (n-butyrate and acetate), followed by unknown non-acidified soluble organics while hydrogen biogas formed a minor portion. As the HRT decreased from 4- to 2-day, it was observed that the proportion of unknown non-VFA soluble organics decreased from 13% to 4%. Overall, 65 to 70% of the influent organic matter remained as insoluble particulate organic matter at the four HRTs tested.

In the case of the mesophilic reactor, the influent soluble organics were completely acidified to VFAs by the acidogenic bacteria at the shorter HRT of 1- and 2-day while at 3- and 4-day HRT, 2 to 3 % of the soluble organics remained as other unidentified non-VFA soluble organics. VFA made up the largest group of the soluble organic fractions. In contrast to the thermophilic effluent, significantly higher proportions of propionate, n-valerate and caproate were present which coincided with negligible hydrogen gas production. Overall, 53 to 65% of the influent organic matter remained as insoluble particulate organic matter.

Reactor performance in the conversion of organic carbon compounds

Table 5.8 compares the organic carbon conversion of the thermophilic and mesophilic reactors at the four HRT tested.

Table 5.8. Extent of net hydrolysis (solubilisation), net acidification and hydrogenogenesis in the thermophilic (T) and mesophilic (M) acidogenic anaerobic reactors

Reactor	HRT (d)	% net hydrolysis	% net acidification	% net hydrogen production
T	1	11 (2)	25 (5)	2 (1)
	2	14 (2)	28 (3)	3 (1)
	3	14 (3)	19 (3)	3 (1)
	4	6 (3)	17 (1)	1 (0)
M	1	16 (3)	35 (4)	0
	2	24 (3)	46 (5)	0
	3	18 (3)	32 (5)	0
	4	21 (4)	45 (6)	0

Data are mean values (± standard deviation)

Statistical t-test analysis ($p<0.05$) of the data indicated that net hydrolysis of the influent particulate organics in the thermophilic reactor was highest at 2- and 3-day HRT, while net acidification of the soluble organics was highest at 2-day HRT. In the case of the mesophilic reactor, both net hydrolysis of the influent particulate organics and net acidification of the soluble organics were highest ($p<0.05$) at 2-day HRT. The overall data show that mesophilic acidogenic reactor hydrolysed and acidified significantly ($p<0.05$) more influent total organic matter than the thermophilic acidogenic reactor.

Anaerobic microbial populations

To gain an insight into the levels of anaerobic microorganisms present in the reactors during 'steady-state', effluents were sampled for FISH analysis (method as described in section 3.2.1.12). Domain-specific ARC-915 and EUBMIX oligonucleotide probes were used to target and quantify the viable methanogenic *archaea* and fermentative bacteria populations. Table 5.9 gives the microbial FISH results of the thermophilic and mesophilic *archaea* and *bacteria* populations in the effluents.

Table 5.9. Quantification of thermophilic (T) and mesophilic (M) anaerobic microorganisms in the acidogenic reactor effluents by FISH method

Reactor	HRT (d)	Archaea (cells/ml)	Bacteria (cells/ml)	Total (cells/ml)	Archaea (%)	Bacteria (%)
T	1	$(8.95 \pm 1.25) \times 10^8$	$(3.19 \pm 0.09) \times 10^8$	$(1.21 \pm 0.13) \times 10^9$	74 (2)	26 (2)
	2	$(7.33 \pm 0.66) \times 10^8$	$(2.53 \pm 0.63) \times 10^8$	$(9.86 \pm 0.02) \times 10^8$	74 (6)	26 (6)
	3	$(6.49 \pm 0.78) \times 10^8$	$(2.38 \pm 0.20) \times 10^8$	$(8.36 \pm 0.57) \times 10^8$	73 (4)	27 (4)
	4	$(8.19 \pm 2.34) \times 10^8$	$(2.33 \pm 0.19) \times 10^8$	$(1.05 \pm 0.26) \times 10^9$	77 (4)	23 (4)
M	1	$(8.25 \pm 0.23) \times 10^8$	$(3.24 \pm 0.04) \times 10^8$	$(1.15 \pm 0.02) \times 10^9$	72 (1)	28 (1)
	2	$(7.07 \pm 0.31) \times 10^8$	$(4.30 \pm 0.54) \times 10^8$	$(1.14 \pm 0.09) \times 10^9$	62 (2)	38 (2)
	3	$(8.68 \pm 0.89) \times 10^8$	$(4.58 \pm 0.06) \times 10^8$	$(1.33 \pm 0.08) \times 10^9$	65 (3)	35 (3)
	4	$(1.12 \pm 0.17) \times 10^9$	$(3.35 \pm 1.15) \times 10^8$	$(1.45 \pm 0.06) \times 10^9$	77 (9)	23 (9)

Data are mean values of replicate measurements (± standard deviation)

Surprisingly, the viable anaerobic *archaea* population whose methanogenic activities had been completely inhibited was found to be greater than the bacteria population (hydrolytic, acidogenic and acetogenic bacteria). Most of the fluorescent cells

targeted by the methanogen-specific ARC915 probe were observed to be very tiny. Not surprisingly, no positive fluorescence signals with ARC-915 for methanogens and EUBMIX for bacteria apart from a few large non-cell auto-fluorescent particles were observed in the synthetic wastewaters which were prepared from commercial pig feed ground pellets in clean distilled water and kept chilled throughout the test runs. The total lack of initial acidification of the soluble organics in the feedwater (Table 5.4) was further evidence that the prepared synthetic wastewater did not harbour initial anaerobic micro-organisms prior to seeding. Given that anaerobic municipal wastewater sludge was used to inoculate the synthetic influent, it was therefore not surprising that micro-organisms were found in the reactor effluent.

The significantly higher levels of mesophilic bacteria, particularly at 2- and 3-day HRT compared to the thermophilic effluent corresponded with the higher performance of the mesophilic acididogenic reactor in the hydrolysis and acidification of the organic carbon compounds (Figure 5.2).

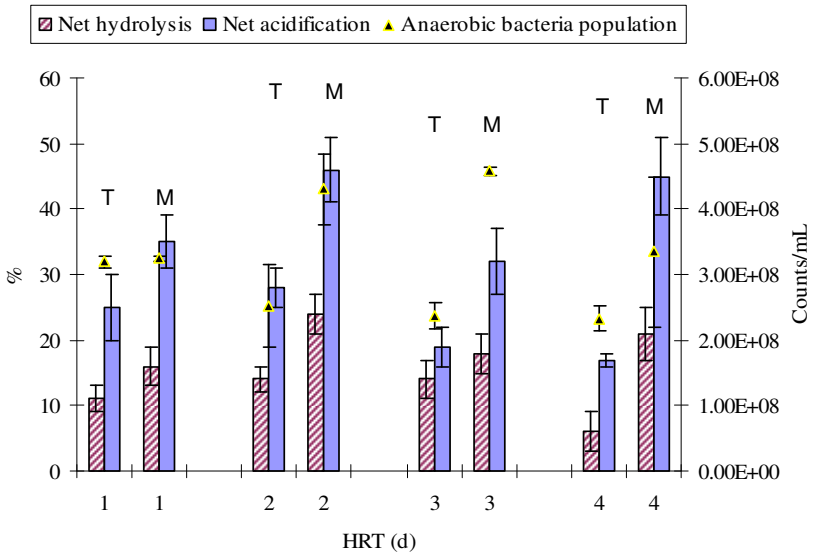
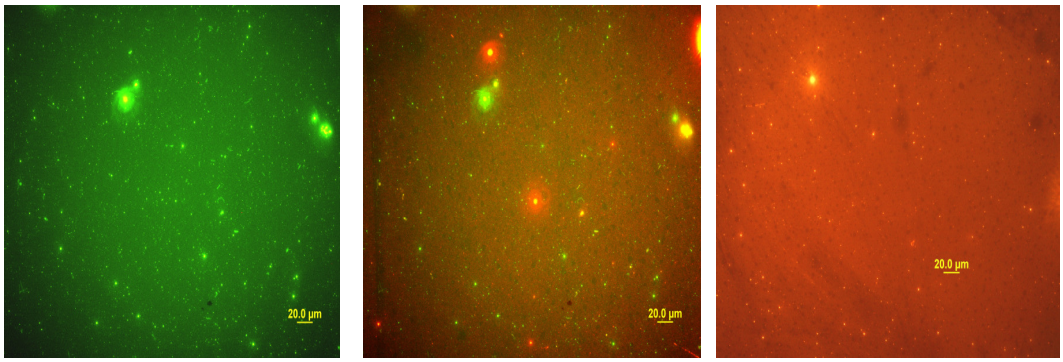


Figure 5.2. Comparison of the thermophilic (T) and mesophilic (M) organics conversion performance in relation to anaerobic bacteria population (error bars indicate standard deviations)

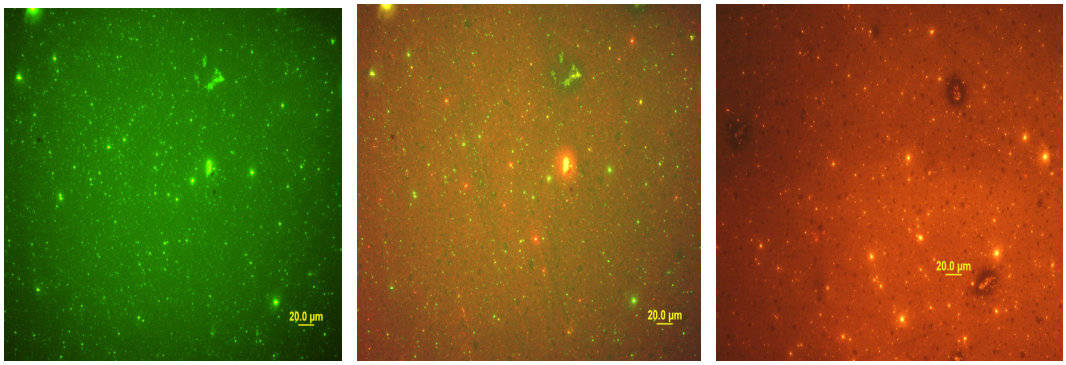
Figures 5.3 (a), (b), (c) and 5.4 (a), (b), (c) show the fluorescent images of *archaea*, *archaea* plus low-GC bacteria and *bacteria* in the thermophilic and mesophilic effluents respectively at 2-d HRT.

(a) (b) (c)



Figures 5.3 (a), (b) and (c). Fluorescent images of archaea (green), archaea (green) plus low-GC bacteria (red) and fermentative bacteria (red) respectively in the thermophilic acidogenic reactor

(a) (b) (c)



Figures 5.4 (a), (b) and (c). Fluorescent images of archaea (green), archaea (green) plus low-GC bacteria (red) and fermentative bacteria (red) respectively in mesophilic acid-phase reactor

To identify and quantify the phylogenetic types of fermentative bacteria, group-specific oligonucleotide probes targeting alpha-, beta- and gamma-proteobacteria as well as the low- and high-GC bacteria were used. The abundances of specific groups of bacteria were expressed as percentages of the *bacteria* domain. Figure 5.5 illustrates the distribution of phylogenetic bacteria groups at the optimum 2-d HRT.

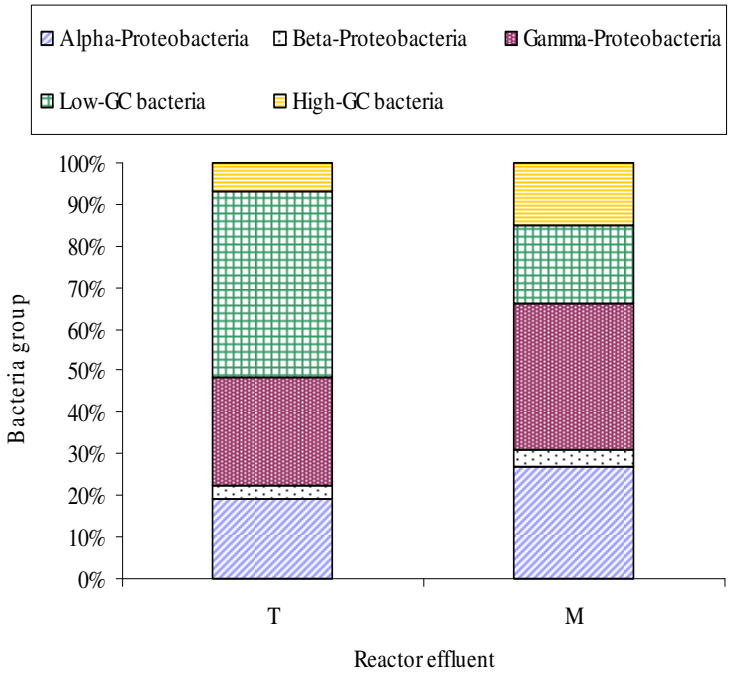


Figure 5.5. Distribution of phylogenetic domains of bacteria in the thermophilic (T) and mesophilic (M) efluents at 2-d HRT

Figure 5.5 shows the thermophilic bacteria were made up largely of low-GC bacteria group. This group covers the spore-forming, hydrogen-producing *Bacillus* and pathogenic *Clostridium* genera amongst others, followed by gamma-proteobacteria, alpha-proteobacteria, high-GC bacteria and beta-proteobacteria in decreasing proportions. The mesophilic bacteria were made up largely of gamma-proteobacteria, followed by alpha-proteobacteria, low-GC bacteria, high-GC bacteria and beta-proteobacteria in decreasing proportions. Appendices 1 and 2 list the bacteria species that belong to the phylogenetic groups of Gram-negative proteobacteria and Gram-positive G+C bacteria respectively.

5.4. DISCUSSION

The reactor performance data (Table 5.8) clearly show the mesophilic (37^{o}C) acidogenic reactor hydrolysed and acidified significantly more influent particulate and soluble organics than the thermophilic (55^{o}C) acidogcnic reactor. The performance results mirrored earlier batch vial experiments (sections 4.3.1 and 4.3.2) but differed with the findings of Guerrero *et al.* (1999) and Yu *et al.* (2002a). Guerrero *et al.* (1999) reported higher solubilisation and acidification of complex

real fishmeal wastewater rich in suspended organic solids and proteins in the CSTR thermophilic reactor (55^{o}C) than the mesophilic reactor (37^{o}C). In contrast, Yu *et al.* (2002a) concluded that there were no differences in the organics acidification of complex synthetic dairy wastewater prepared from soluble full cream powdered milk between mesophilic (37^{o}C) and thermophilic (55^{o}C) UASB acidogenic reactors. As suggested earlier in section 4.4.1, differences in wastewater composition, reactor configuration and the subsequent microbial types selected could all contribute to the differences in findings between all these studies. It was noted that for the adjusted influent pH of 8.0, pH of both thermophilic and mesophilic effluents were lower than the batch experiments (Tables 4.2 and 4.3).

While microbial results were not available in previous studies (Guerrero *et al.*, 1999 and Yu *et al.*, 2002a), this study found higher levels of bacteria in the mesophilic acid-phase CSTR than the thermophilic counterpart at the optimum 2-day HRT for organics solubilisation and acidification (Figure 5.2). The detection of viable *archaea* cells by the fluorescently-labelled *archaea*-specific oligonucleotide probe showed that the methanogens were not being washed out at 1- to 4-day HRT. The lack of methane in the small amount of biogas produced suggested that the fast-growing hydrogen-consuming methanogens with a short doubling time of 4 to 11 hours and the slow-growing acetate-consuming methanogens with a doubling time of 2 to 3 days (section 2.2.4 in Chapter 2) had been effectively inhibited in the acidic-phase reactor effluent. One surprising observation is that the proportion of *archaea* was greater than the proportion of bacteria in the total viable cells. Given that the viable methanogens were inhibited from consuming the methanogenic substrates and thus assumed to be inactive or dormant, one would expect the active *bacteria* numbers to greatly outnumber the inactive *archaea*. Two possible explanations for the observed anomaly are that: 1) the *bacterial* cell numbers were underestimated due to probe permeability problem with the cell membrane of some bacterial groups (Table 2.6 in Chapter 2); and 2) the *archaeal* cell numbers were overestimated due to enhanced sensitivity of the image processing software in detecting inactive cells with weak fluorescence intensity that would otherwise not have been visualised on the images captured with the digital camera. The viable *archaea* counts highlight the limitation of FISH molecular method on its inability to differentiate active viable cells from inactive (dormant) viable cells that are inhibited by the unfavourable

acidic environment in the acid-phase anaerobic reactor. Wagner *et al*. (2003) has highlighted that FISH method does not provide indications on the physiological activity and function of the probe-targeted cells. Nevertheless, more research effort is required in this area to clarify the cause of the observed anomaly in regards to the *archaea*: *bacteria* ratio particularly in a methanogens-stressed environment.

The COD material balance of thermophilic (Figure 5.1 (b)) and mesophilic reactor effluents (Figures 5.1 (d)) clearly highlight that while the mesophilic acid-phase reactor hydrolysed about 10% more than the thermophilic reactor at the optimum HRT of 2-day, the majority of the influent organic matter (over 55%) was not being solubilised and remained in particulate form. Although total alkalinity was not measured, the rapid drop of the adjusted influent initial pH of 8 to final pH of 3.9 and 4.4 in the mesophilic and thermophilic acid-phase reactors respectively clearly indicated that the synthetic wastewater lacked natural buffering capacity to counteract the VFAs produced during fermentation. It is possible that the rapid drop in the effluent pH could have retarded further hydrolysis of the influent particulate organics (carbohydrates, proteins and lipids) to soluble forms (sugars, amino acids, LCFAs). It was reported that at pH below 5, acidification of carbohydrate was reduced by five-fold while hydrolysis of lipid to LCFA was reduced by 2-fold (Hanaki *et al*., 1987). Yu and Fang (2002) observed that the conversion of carbohydrate, protein and lipid increased with increasing pH from 5.5 to 6.5. However, methanogenesis began to set in at pH above 5.5. It appears that pH control of the acid-phase reactors to between pH 5 and 5.5 might be necessary to further increase the conversion of the influent particulate organics to soluble, acidified forms.

The observed differences in acidified products distribution between thermophilic (Figure 5.1 (b)) and mesophilic (Figure 5.1 (d)) acid-phase reactors suggested that temperature had a greater influence over HRT on the type of fermentation products formed. The product composition of the thermophilic effluent resembled a dominant butyric acid fermentation which is characterised by the production of butyrate, acetate, hydrogen and carbon dioxide as the main fermentation products (Barton, 2005; Yu and Fang, 2001). In contrast, the product composition of the mesophilic effluent resembled a mix of propionate and butyrate fermentation which is

characterised by the formation of propionate, acetate, butyrate, valerate and insignificant hydrogen production.

Interestingly, the propionate concentration in the thermophilic acid-phase reactor effluent was significantly less than the mesophilic counterpart. This observation was in stark contrast to the high propionate concentration commonly encountered in the effluent of the thermophilic anaerobic single-stage reactor (Kim *et al*., 2002; Fang and Chung, 1999; Kaiser *et al*., 1995). The lower propionate concentration in the thermophilic acid-phase reactor effluent could possibly be due to different enrichment that developed under thermophilic conditions and/or the effluent had lower dissolved hydrogen concentration as a result of the release of free hydrogen biogas which facilitated the efficient oxidation of propionate and higher carbon-chain VFAs in the anaerobic reactor. Although numerous articles cite high hydrogen partial pressure to favour the formation of propionate and higher molecular weight organic acids (Harper and Pohland, 1987; Novaes, 1986), it is unclear whether it means dissolved hydrogen concentration in the liquid or free hydrogen concentration in the biogas. As methanogenesis of propionate is the most difficult intermediate metabolic product to degrade compared to acetate and butyrate (de Bok *et al*., 2004), it appeared that the thermophilic reactor effluent in this study could provide more favourable carbon and energy source for the second-phase methane reactor as it contained much lower propionic acid concentration than the mesophilic reactor effluent.

The higher proportions of unidentified soluble organics in the thermophilic effluent (Figure 5.1 (b)) at longer HRT of 3 and 4 days compared to the mesophilic effluent (Figure 5.1 (d) was probably due to increased microbial inhibition by the combined effects of high temperature and acidic effluent pH. The unidentified soluble organics could consist of unused substrates such as sugars, amino acids and long-chain fatty acids as well as other metabolic products such as cell lysis, lactic acid, formic acid, acetone and acetaldehyde (Barton, 2005; Yu and Fang, 2001; Elefsiniotis *et al.*, 1996; Eastman and Ferguson, 1981). These organic compounds could not be measured with the gas chromatography column for VFA analysis.

A key characteristic of acid-phase anaerobic digestion is its very low production of biogas which is comprised largely of carbon dioxide, nitrogen and hydrogen as by-

products of the acidogenesis step of the fermentation. Ideally, methane production should be negligible in order to conserve the VFAs as carbon and energy sources for the methane reactor or the enhanced biological nutrient removal processes. However, in practice, variable amounts of methane have been reported in acid-phase anaerobic reactors treating diluted real primary sludge (Miron *et al.*, 2000; Elefsiniotis *et al.*, 1996; Eastman and Ferguson, 1981) and synthetic wastewater (Yu and Fang, 2002; Fang and Yu, 2001) due to either inadequate phase separation or inadequate pH suppression of the methanogens.

In this study, methane production was completely suppressed by the acidic reactor effluent as evidenced by its absence in the biogas produced from the thermophilic and mesophilic acid-phase reactors (Table 5.7). The significantly higher hydrogen yield from the thermophilic acid reactor compared to the yield from mesophilic acid reactor was in agreement with Yu *et al.* (2002). The higher hydrogen yield coincided with the significantly lower propionate, valerate and caproate concentrations in the thermophilic reactor effluent (Table 5.6). This observation seemingly agreed with Harper and Pohland (1987) who recommended that hydrogen be removed from the liquid phase as free gas washout to enable the efficient oxidation of propionate and higher carbon-chain VFAs in the anaerobic reactor.

With the organic matter in the wastewater remaining largely conserved as solubilised compounds in the acid-phase anaerobic digestion, the observed large effluent total solids (TS) and volatile solids (VS) reduction or removal data in Table 5.3 particularly at mesophilic temperature, did not reflect organics degradation as methanogenesis was inhibited in the thermophilic and mesophilic acid-phase reactors. The large reductions in effluent TS and VS can be explained by the fact that in TS measurement, dissolved volatile organic solids with low boiling points such as acetone (b.p. 56^{o}C), formic acid (b.p. 100-101^{o}C), ethanol (b.p. 68^{o}C) and other low-boiling metabolites, if present will be lost during oven-drying at 105^{o}C. The resultant reduction in effluent total solids then affected the subsequent VS measurement. Pind *et al.* (2003) have reported that between 78 and 84% of the spiked VFA in cattle manure was lost in TS and VS measurements. Their findings concluded that TS and VS removal data when used on samples containing large

amount of VFAs could yield erroneous information in regards to the reactor digestion performance.

For TSS and VSS measurements, as the dissolved volatile solids have been removed from the suspended solids, TSS and VSS removal data are considered more meaningful than TS and VS removal data as they can provide complementary information on the extent of solubilisation of the influent particulate organic matter. However, problem with sampling particulate-containing wastewater for representative solids measurements can make results interpretation difficult. This was encountered with the effluent total COD measurements in this study. In addition, VSS data which is commonly used to represent microorganisms (biomass) in wastewater treatment can be severely distorted by the suspended organic substrates present in the particulate-containing wastewater (Yu *et al*., 2002b).

During anaerobic degradation of proteinaceous materials, ammonia is generated alongside VFAs production (McInerney, 1988). The higher net increase in ammonium-nitrogen concentrations (Table 5.1) observed in the thermophilic acid-reactor effluent compared to the mesophilic effluent indicated that thermophilic temperature was more effective than mesophilic temperature in solubilising and acidifying some of the influent protein component in the complex substrate. This observation was consistent with the findings of Penaud *et al*. (1997) and Guerrero *et al.* (1999) that increased temperature enhanced solubilisation and acidification of proteins in the real complex pharmaceutical and fishmeal wastewaters respectively. In the case of the soluble phosphorus, the comparable net increased concentrations between thermophilic and mesophilic effluents suggested that temperature was not responsible for the release of organically-bound phosphorus in the influents. In enhanced biological nutrient removal systems, volatile fatty acids are known to promote phosphorus release from cells under anaerobic conditions (Skalsky and Daigger, 1995).

5.5. CONCLUSIONS

The results of the semi-continuous reactor study indicated that maximum hydrolysis and acidification of the influent organic matter could be obtained at a reactor HRT as short as 2-day for both the mesophilic and thermophilic acid-phase anaerobic

reactors. The significance of the short HRT is that the required size of the acid-phase anaerobic reactor can be cost-effectively reduced and still provides optimum fermentation of the particulate-containing wastewater. The significantly (p<0.05) higher percentages of net hydrolysis and acidification of the organics which coincided with higher number of bacteria at mesophilic temperature (37^{o}C) over thermophilic temperature (55^{o}C) supported the adoption of mesophilic acid-phase reactor as it was more cost-effective in terms of lower energy requirement for heating than thermophilic acid-phase reactor. However, thermophilic reactor effluent was observed to contain lesser concentrations of propionic acid which made it more energetically favourable for bioconversion to methane in the second-phase methane anaerobic reactor. Although its ammonium-nitrogen concentration (5 to 12 mg N/L) was higher than the mesophilic effluent (0.2 to 0.4 mg N/L), it was not expected to pose any problems to the methanogenic microorganisms as the concentration was way below the inhibitory value of 560-568 mg-N/L reported by Gallert and Winter (1997) to cause 50% inhibition of methanogenic activity at pH 7.6.

One important observation of the reactors' organics conversion performance is that a large fraction of the influent organic matter (over 55%) remained in particulate form and was thus not available for conversion to VFAs. With the effluent pH dropping to below literature optimum of pH 5.5-6.0 during semi-continuous reactor treatment, automated pH control would be necessary to further enhance organics solubilisation and acidification.

CHAPTER 6
SEMI-CONTINUOUS FIRST-STAGE (PHASE) ANAEROBIC DIGESTION OF RAW PIGGERY WASTEWATER AT LOW HYDRAULIC RETENTION TIME

6.1. INTRODUCTION

At the project demonstration site in Roseworthy Campus of the University of Adelaide, South Australia, raw piggery wastewater which is made up of a mixture of pig excrements (faeces and urine), droppings of grain-based rations and wash water is collected from a 500-sow piggery holding sump and treated in a pilot-scale two-stage (phase) anaerobic digester system. The latter is comprised of two daily-fed 200-L thermophilic first-stage acidogenic continuously-stirred tank reactors (CSTR) operating at 55^{o}C and 7-day hydraulic retention time without pH control in series with four 225-L second-stage polybag methane digesters operating at 23-day HRT and ambient conditions. The primary aims of the thermophilic acidogenic reactors are to enhance pathogens kill and to maximise the bioconversion of organic carbon compounds to volatile fatty acids for feed to the second-stage methane reactors.

During the course of the pilot-scale reactor operations, it was reported that the total volatile fatty acids produced in the first-phase thermophilic acidogenic reactors were rather low, at less than 1 g/L VFA as COD (Appendix 3.2). In addition, the composition of the biogas produced was not known with certainty at the time. In view of these unanswered questions in regards to the fermentation performance of the pilot-scale acidogenic reactors, it was decided to abandon the use of synthetic complex wastewater at this stage and to adopt the same piggery wastewater as SARDI to allow meaningful and relevant laboratory-scale reactor experiments to be designed and conducted. The change in research direction created an opportunity to conduct comparison study of the acid-phase anaerobic digestion of real piggery wastewater with the previous synthetic complex wastewater. To guide the design of appropriate acid-phase reactor experiments with the raw piggery wastewater, the following research questions were framed to seek some clarifications on the pilot-scale acidogenic digestion of piggery wastewater:

- Why did the raw piggery wastewater not acidify in the pilot-scale thermophilic first-stage anaerobic reactors unlike the synthetic complex wastewater?

- Which of the operational factors (pH, feed concentration or organic loading rate, temperature, hydraulic retention time) would best facilitate the piggery wastewater to acidify in the first-stage acidogenic anaerobic reactor?

It was hypothesised that if the hydraulic retention time of the pilot-scale first- phase reactor was reduced from 7 days down to 2 days, total VFA production would increase and the wastewater pH would drop due to the fast growing acidogenic bacteria dominating over the slow growing VFA-consuming acetogenic and methanogenic microorganisms. The choice of 2-day HRT was based on previous acidogenic reactor experiments which found it to be optimum for the hydrolysis and acidification of the synthetic complex organic wastewater. In addition, numerous studies had demonstrated that short HRT of $\leq$ 24 hours facilitates the preferential selection of the fast-growing VFA-producing acidogens over the slow-growing VFA-consuming synthropic acetogens and methanogens in the acidogenic reactor (Lettinga and Hushoff Pol, 1986; Guerrero *et al*., 1999; Yu and Fang, 2001). It was also hypothesised that through the combined pH reduction of the influent, low reactor HRT and high influent concentration, organics conversion performance of the first-phase acidogenic reactor would be enhanced and resulted in maximum volatile fatty acids production.

In order to test these hypotheses, laboratory-scale reactor experiments were designed with the following objectives:

- To investigate the effects of piggery influent concentrations (diluted low-strength, diluted mid-strength and undiluted high strength) and pH reduction on the fermentation performance of thermophilic acid-phase anerobic reactor at a shorter HRT of 2 days as opposed to 7 days used in the pilot-scale acidogenic reactor; and

- To take the opportunity to compare thermophilic and mesophilic fermentation results of the low-strength piggery wastewater with the previous acid-phase

reactor experiments on the low-strength synthetic complex wastewater at 2-day HRT.

This chapter details the experimental designs and findings of the laboratory-scale first-stage thermophilic (55^{o}C) and mesophilic (37^{o}C) anaerobic reactors treating low-strength piggery wastewater without pH reduction at 2-day hydraulic retention time; and the first-stage thermophilic (55^{o}C) anaerobic reactors treating medium-strength and high-strength (undiluted) piggery wastewater with and without pH reduction on the feedwater at 2-day hydraulic retention time.

6.2. MATERIALS AND METHODS

6.2.1. Semi-continuous first-stage CSTR anaerobic reactors treating low-strength piggery wastewater without pH reduction at thermophilic (55^{o}C) and mesophilic (37^{o}C) at 2-day hydraulic retention time

6.2.1.1. Objectives

The objectives of these experiments were: 1) to examine the extent of organics solubilisation and acidification of the low TCOD strength piggery wastewater in the thermophilic (55^{o}C) and mesophilic (37^{o}C) acidogenic anaerobic reactors at 2-day HRT; and 2) to compare thermophilic and mesophilic acidogenic digestion of the low-strength piggery wastewater with the previous low-strength synthetic complex wastewater at 2-day HRT.

6.2.1.2. Method

The thermophilic (55^{o}C) and mesophilic (37^{o}C) anaerobic acidogenic reactors as described in section 3.2.2 (Chapter 3) were seeded with their respective chilled anaerobic cultures from the earlier experiments to a working volume of 0.9 L. The reactors were gradually brought up to the required thermophilic and mesophilic temperatures over two days. While waiting for the raw piggery wastewater to arrive from SARDI, the reactors were drained and fed semi-continuously at 2-day HRT for 53 days with the low-strength piggery wastewater prepared from pig manure collected from the solids removal separator of Murdoch University's piggery farm.

Following the arrival of raw piggery wastewater from SARDI, the piggery wastewater was diluted with deionised water to the targeted total COD feed concentration of 4-5 g/L to allow for comparison with the earlier reactor experiment with the synthetic complex wastewater. The reactors were drained and fed ten times daily at 2.4 hr interval to achieve the required 2-day HRT with the low strength piggery wastewater. Four volumes of hydraulic retention time turnover were allowed to elapse to allow for steady state conditions to be achieved before the effluent and biogas were sampled and analysed for eight consecutive days. Replicate gas composition was sampled and analysed for hydrogen, methane and carbon dioxide while triplicate feedwaters and effluents were tested for pH, alkalinity, total and soluble COD, total and volatile suspended solids, C2 to C6 volatile fatty acids, ammonia-nitrogen, soluble phosphorus and replicate samples were tested for fluorescence *in situ* hybridisation (FISH) and T-RFLP microbial profiling analysis.

The assessment of reactor conversion performance or efficiency was based on the extent of net hydrolysis and net acidification as per section 5.2.2 in Chapter 5 that took into account the changes in soluble organics, volatile fatty acids and methane production.

The extent of methanogenesis is calculated as follows:
Degree of methanogenesis (%) = (methane-COD/influent TCOD) 100*

Free ammonia concentration was calculated from the following formula (Hansen *et al*., 1998):
$NH_3\text{-}N = (\text{Total ammonium-nitrogen}) \times (1 + 10^{-pH}/10^{-(0.09018 + 2729.92/T)})^{-1}$
Where T = temperature (Kelvin); and
Total ammonium-nitrogen = ammonia- and ammonium-nitrogen

The significance of differences in mean data was statistically compared using Excel statistical t-test data analysis software at 95% confidence level.

6.2.2. Semi-continuous thermophilic first-stage CSTR reactors (55^{o}C) treating pH-unadjusted and pH-reduced medium- and high-strength piggery wastewater at 2-day hydraulic retention time

6.2.2.1. Objectives

The objectives of these experiments were: 1) to investigate the effects of increasing piggery influent TCOD concentrations or organic loading rates (TCOD/L/d) on the extent of organics solubilisation and acidification of the piggery wastewater in the thermophilic (55^{o}C) acidogenic reactor at 2-day HRT; 2) to investigate the effect of pH reduction of the piggery wastewater to pH 5.5 on the extent of organics solubilisation and acidification in the thermophilic (55^{o}C) acidogenic reactors at 2-day HRT; and 3) to compare the organics conversion efficiency of the piggery wastewaters with and without pH reduction.

6.2.2.2. Method

Two thermophilic CSTRs described in section 3.2.2 of Chapter 3 were seeded with 0.9 L of the low-strength digested piggery effluents from previous thermophilic reactor experiment. One reactor was fed with diluted medium-strength piggery wastewater without pH reduction while the other reactor was fed with diluted medium-strength piggery wastewater with pH reduction to 5.5. This pH was selected as it had been demonstrated to produce maximum volatile fatty acids in the first-phase acidogenic reactors treating complex wastewaters (Yu and Fang, 2001; Elefsiniotis *et al.*, 1996).

The chilled raw piggery wastewater was sieved with 1 mm mesh screen to remove coarse food particles as they blocked the pump tubing in the earlier reactor experiment (section 6.2.1). To prepare the medium-strength wastewater, the sieved piggery wastewater was diluted with deionised water to give the targeted TCOD concentration. The pH-reduced medium-strength wastewater was prepared by adding concentrated hydrochloric acid to the piggery influent rather than the reactor wastewater to avoid foaming-related spillage from the reactor. The semi-continuously pumped CSTR reactors were drained and fed ten times daily at 2.4 hr interval to achieve the required 2-day HRT with the medium-strength piggery

wastewaters. After four weeks of elapsed time to allow steady state conditions to be achieved, piggery influents and reactor effluents as well as biogas were sampled and analysed as per section 6.2.1.2 for eight consecutive days. Following completion of the medium-strength anaerobic digestion experiments, the reactors were fed with their respective undiluted high-strength piggery wastewaters with and without pH reduction to start the next acid-phase digestion experiments. The same procedures of reactor operations, sampling, analysis and data-processing as the earlier experiments with the medium-strength wastewaters were adopted.

6.3. RESULTS

6.3.1. Semi-continuous first-stage thermophilic (55^{o}C) and mesophilic (37^{o}C) CSTR anaerobic reactors treating low-strength piggery wastewaters without pH reduction at 2-day HRT

6.3.1.1. Thermophilic and mesophilic anaerobic treatments of low-strength piggery wastewater

pH, total alkalinity, ammonium-nitrogen, free ammonia and soluble phosphorus

Table 6.1 gives the pH, total alkalinity, ionised ammonium-nitrogen, unionised dissolved ammonia and soluble phosphorus concentrations in the piggery influents and effluents of the thermophilic and mesophilic anaerobic reactors.

Table 6.1. pH, total alkalinity, ammonium-nitrogen and free ammonia concentrations of the low-strength influents and effluents at 2-d HRT

Piggery wastewater	pH	Total alkalinity (mg $CaCO_3$/L	NH_4^+-N (mg/L)	Free NH_3-N (mg/L)	Soluble phosphorus (mg/L)
Influent to T-reactor	7.6 (0.1)	2190 (170)	690 (99)	3 (1)	46
T-effluent	7.7 (0.2)	2505 (64)	690 (61)	105 (27)	43 (6)
Influent to M-reactor	7.5 (0)	2100 (297)	655 (7)	2 (0)	45
M-effluent	7.6 (0.1)	2475 (106)	650 (46)	31 (11)	49 (2)

Data are mean values (± standard deviation) T (thermophilic) M (mesophilic)

With the exception of free ammonia concentrations in the effluents, all the other four parameters remained relatively constant after thermophilic or mesophilic anaerobic treatment of the piggery wastewater. Free ammonia concentration was observed to be three-fold higher in the thermophilic effluent than the mesophilic effluent due to the higher reactor temperature which increased the proportion of unionised free ammonia. For the same reason, thermophilic and mesophilic effluents had significantly higher free ammonia concentrations than the chilled influents fed to the reactors.

Total and volatile suspended solids (TSS and VSS)

Table 6.2 gives the total and volatile suspended solid concentrations in the influents and effluents of the thermophilic and mesophilic anaerobic reactors. The high percentage influent VSS/TSS values of over 97% show the suspended solids to consist almost entirely of organic matter.

Table 6.2. Total and volatile suspended solids in the low-strength influents and effluents at 2-d HRT

Piggery wastewater	TSS (g/L)	VSS (g/L)	VSS/TSS	VSS removal (%)
Influent to T-reactor	1.7 (0.2)	1.6 (0.2)	98 (12)	
T-effluent	1.5 (0.5)	1.2 (0.3)		33 (14)
Influent to M-reactor	1.6 (0.2)	1.6 (0.2)	97 (5)	
M-effluent	1.7 (0.4)	1.3 (0.2)		20 (8)

Data are mean values (± standard deviation) T (thermophilic) M (mesophilic)

Statistical t-test comparison of the VSS removal data indicated the thermophilic anaerobic reactor to solubilise significantly ($p<0.05$) more particulate organic matter compared to the mesophilic anaerobic reactor. The results were in agreement with the calculated net extent of solubilisation (hydrolysis) data given in Table 6.6.

COD, volatile fatty acids and biogas

Table 6.3 gives the COD (total and soluble) and total VFA-COD data of the low-strength piggery influents and effluents of the thermophilic and mesophilic reactors. The percentage ratio of soluble COD/total COD was calculated to determine the amount of organics that had solubilised in the piggery influent as received. Similarly,

the percentage ratio of total VFA-COD/soluble COD was calculated to determine the amount of soluble organics that had acidified to volatile fatty acids in the piggery influent as received.

Table 6.3. Chemical oxygen demand (total and soluble) and total VFA concentrations of the low-strength piggery influents and reactor effluents at 2-d HRT

Piggery wastewater	Total COD (mg/L)	Soluble COD (mg/L)	Total VFA (mg COD/L)	SCOD/TCOD (%)	TVFA-COD/SCOD (%)
Influent to T-reactor	4449 (211)	2697 (139)	2893 (96)	61 (4)	107 (4)
T-effluent	4384 (404)	2750 (122)	2209 (187)		
Influent to M-reactor	5235 (17)	2542 (149)	2438 (272)	49 (3)	96 (12)
M-effluent	5004 (393)	2631 (99)	2132 (92)		

Data are mean values (± standard deviation) T (thermophilic) M (mesophilic)

Despite the spatial variation of the particulate-containing piggery wastewater which accounted for the variable total COD measured, the influent SCOD:TCOD data show over half of the total organic matter in the diluted piggery wastewater was already present in soluble form. The soluble organic fraction had also completely or almost completely been fermented to VFAs by the indigenous acidogenic bacteria as shown by the high TVFA:SCOD ratio of around 100%.

Figure 6.1 illustrates the COD reductions of the thermophilic and mesophilic effluents. While total COD and soluble COD reduction data showed there were no differences in COD removal between thermophilic and mesophilic first-stage reactors, total VFA-COD reduction data indicated that the thermophilic reactor removed significantly more VFAs than mesophilic reactor. The observed inconsistencies between TCOD, SCOD and TVFA-COD reductions were attributed to the uneven distribution of particulate organics in the unsieved whole sample and the non-VFA components present in the soluble fraction which distorted the calculated non-specific COD reduction values as opposed to the VFA-specific COD reduction values.

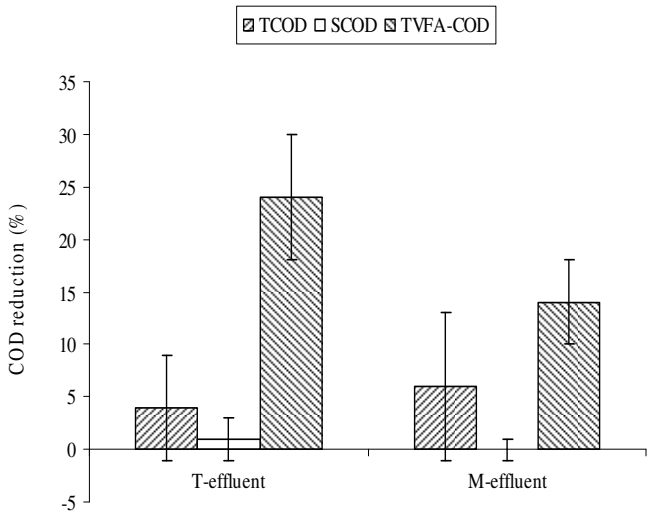


Figure 6.1. COD (total and soluble) and total VFA reductions of thermophilic (T) and mesophilic (M) digested effluents at 2-d HRT (error bars indicate standard deviations)

Table 6.4 shows the influent VFA concentrations of acetate, propionate, n-butyrate and n-valerate were significantly ($p<0.05$) reduced by the thermophilic and mesophilic reactors. However, the isomer forms of butyrate and valerate as well as caproate concentrations were observed to be elevated in the effluents as illustrated by the negative percentage reduction in Figure 6.2.

Table 6.4. Volatile fatty acid concentrations of the low-strength piggery influents and digested effluents at 2-d HRT

Piggery wastewater	Acetate (mg COD/L)	Propionate (mg COD/L)	i-butyrate (mg COD/L)	n-butyrate (mg COD/L)	i-valerate (mg COD/L)	n-valerate (mg COD/L)	Caproate (mg COD/L)
Influent to T-reactor	1086 (46)	639 (11)	127 (19)	437 (12)	292 (7)	234 (25)	78 (4)
T-effluent	741 (79)	474 (59)	158 (17)	243 (119)	335 (16)	170 (28)	88 (7)
Influent to M-reactor	943 (110)	518 (66)	105 (19)	349 (42)	265 (17)	184 (25)	74 (5)
M-effluent	732 (37)	441 (50)	143 (12)	259 (10)	304 (11)	178 (16)	75 (10)

Data are mean values (± standard deviation) T (thermophilic) M (mesophilic)

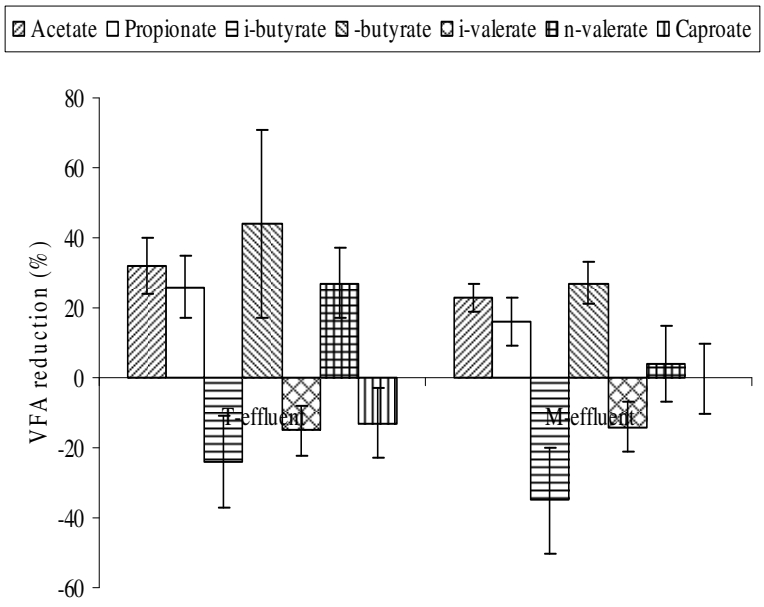


Figure 6.2. Percentage removal of VFA-COD in the thermophilic (T) and mesophilic (M) digested effluents at 2-d HRT (error bars indicate standard deviations)

Table 6.5 gives the biogas composition and actual specific methane yields obtained from the thermophilic and mesophilic first-stage reactors. The biogas contained 58-65% methane and 18-24% carbon dioxide, with the balance assumed to be nitrogen which could not be measured as high-purity nitrogen was used as the GC column carrier gas. The detection of methane indicated that methanogenic *archaea* was present in the piggery effluent despite the low HRT and initial seeding with acidogenic culture.

Table 6.5. Biogas composition and specific methane yields from thermophilic and mesophilic first stage reactors treating low-strength piggery wastewaters

Reactor	TVFA-COD removal rate (g SCOD/L/d)	% methane	% carbon dioxide	Actual specific methane yield (L CH_4/g TVFA-COD removed) at stp
55^{o}C	0.329 (0.098)	65 (3)	24 (2)	0.154 (0.033)
37^{o}C	0.169 (0.063)	58 (2)	14 (1)	0.098 (0.04)

Data are mean values (± standard deviation) Mean of 7 to 8 measurements

stp (standard temperature pressure of 1 atmosphere and 0^{o}C)

It is noted that COD data obtained by wet chemistry standard method is commonly used for calculating the actual specific methane yield due probably to it being easier and cheaper to perform than the gas chromatography and conversion method of

VFA-COD data. However in this study, the TVFA-COD removal data was preferred over direct soluble COD removal data for two reasons. Firstly, the TVFA-COD data is VFA-specific while the SCOD data includes other non-VFA organic materials such as unused soluble substrate, product of cell lysis and extracellular intermediate metabolites (Elefsiniotis *et al*., 1996; Eastman and Ferguson, 1981). Secondly, the mean SCOD removal data obtained were highly inconsistent with the observed total VFA-SCOD destroyed as well as the observed methane production. On the basis of these considerations, it was decided to use the TVFA-COD removal rate data for calculating the actual methane yield and to compare it with the theoretical value of 0.35 L.methane produced per gram of COD degraded or removed (Choi, 2007; de Lemos Chernicharo, 2007).

Table 6.5 shows the actual specific methane yield was significantly ($p<0.05$) higher at 55^{o}C than at 37^{o}C. Both the actual specific methane yields were significantly less than the theoretical value of 0.35 L/g COD removed. Possible factors that contributed to the lower values were measurement errors incurred in biogas and VFA determinations.

COD material balance

To compare the proportions of influent organic matter converted to those in the effluents after thermophilic or mesophilic anaerobic treatment, COD material balance was constructed from the SCOD, VFA-COD and methane–COD components present in the influents and effluents in relation to their respective influent TCOD (Fugure 6.3). Methane-COD (mg COD/L) was obtained by first converting the daily methane production rate (L methane/L/d) to mg methane-COD/L/d using a conversion factor of 2860 mg/L methane (Eastman and Ferguson, 1981) and then multiplying by the HRT (d) (Hanaki *et al*., 1987).

It is clear that after anaerobic treatment in the first-stage reactors, the soluble organic fractions of both thermophilic and mesophilic effluents remained largely as VFAs which was the intention in running the reactors at a short HRT of 2 days in order to minimise VFA losses to methane gas production. Acetate was observed to be the major VFA in the thermophilic and mesophilic influents, followed by propionate, n-butyrate, i-valerate, n-valerate, i-butyrate and caproate in decreasing order. Both the

thermophilic and mesophilic effluents were observed to contain higher proportions of unidentified soluble organics compared to their influents.

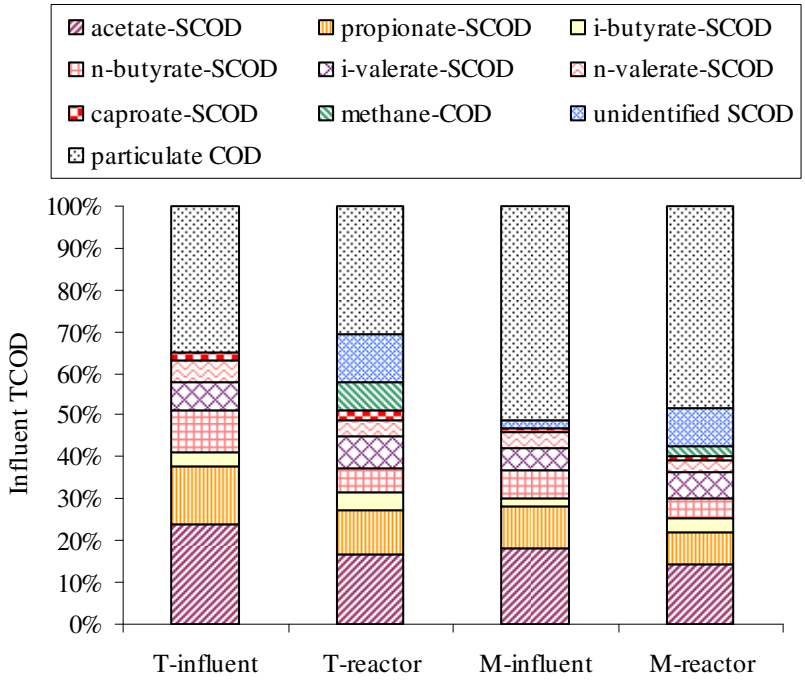


Figure 6.3. COD material balance of the low-strength piggery influents and digested effluents of the thermophilic (T) and meophilic (M) anaerobic reactors

While small net increases in hydrolysis were observed to occur during first-stage thermophilic and mesophilic anaerobic treatments, there were no additional conversions of the soluble organic fractions to VFAs (Table 6.6). Instead, small but significant ($p<0.05$) reduction of the influent VFA concentrations occurred with concurrent methane production. The production of methane clearly indicated that methanogenic microorganisms were present at 2-day HRT. Molecular FISH images of the thermophilic and mesophilic effluents in Figures 6.4 (a) and 6.5 (a) respectively positively confirmed their presence among the anaerobic fermentative bacteria.

Overall, the COD material balance of thermophilic and mesophilic effluents show the proportion of insoluble organics ranged from 31 to 48% as particulate COD while the proportion of soluble organics ranged from 50 to 62% as VFAs, methane and non-VFA components.

Reactor performance in the conversion of organics

Despite the spatial variations in the influent COD concentrations, comparison of the thermophilic and mesophilic reactor performance in organics conversion was based on the net data of hydrolysis and acidification rather than the gross data. The net organics conversion data are given in Table 6.6.

Table 6.6. Comparison of the extent of net hydrolysis, net acidification and methanogenesis in the first-stage anaerobic reactors treating low-strength piggery wastewaters

Parameter	Unit	Thermophilic reactor	Mesophilic reactor
Extent of net hydrolysis	% TCOD fed	8 (3)	4 (3)
Extent of net acidification	% TCOD fed	0	0
Extent of methanogenesis	% TCOD fed	7 (1)	2 (1)

Data are mean values (± standard deviation)

Statistical t-test comparison of the mean net extent of hydrolysis and methanogenesis indicated that the thermophilic first-stage reactor hydrolysed and converted significantly ($p<0.05$) more organic carbon compounds to methane than the mesophilic reactor.

Anaerobic microbial populations

Similar to the previous acidogenic anaerobic reactor experiments on the synthetic complex wastewater in Chapter 5, ARC-915 and EUBMIX domain-specific *archaea* and *bacteria* populations. Table 6.7 gives the FISH microbial results of the thermophilic and mesophilic *archaea* and *bacteria* populations at 2-day HRT.

Table 6.7. Quantification of anaerobic thermophilic and mesophilic microorganisms using 16S rRNA-domain specific FISH probes of ARC-915 for *archaea* and EUBMIX for *bacteria*

Sample	*Archaea* (cells/ml)	*Bacteria* (cells/ml)	Total viable prokaryote (*archaea* + *bacteria*) (cells/ml)	% *Archaea*	% *Bacteria*
FT	$(4.79 \pm 0.04) \times 10^8$	$(5.52 \pm 0.11) \times 10^8$	$(1.03 \pm 0.01) \times 10^9$	46 (0.3)	54 (0.3)
TE	$(8.31 \pm 1.17) \times 10^8$	$(1.27 \pm 0.49) \times 10^9$	$(2.10 \pm 0.60) \times 10^9$	40 (6)	60 (6)
FM	$(4.84 \pm 0.84) \times 10^8$	$(5.37 \pm 0.86) \times 10^8$	$(1.02 \pm 0.17) \times 10^9$	47 (0.4)	53 (0.4)
ME	$(1.52 \pm 0.05) \times 10^9$	$(6.28 \pm 0.24) \times 10^8$	$(2.14 \pm 0.07) \times 10^9$	71 (0.1)	29 (0.1)

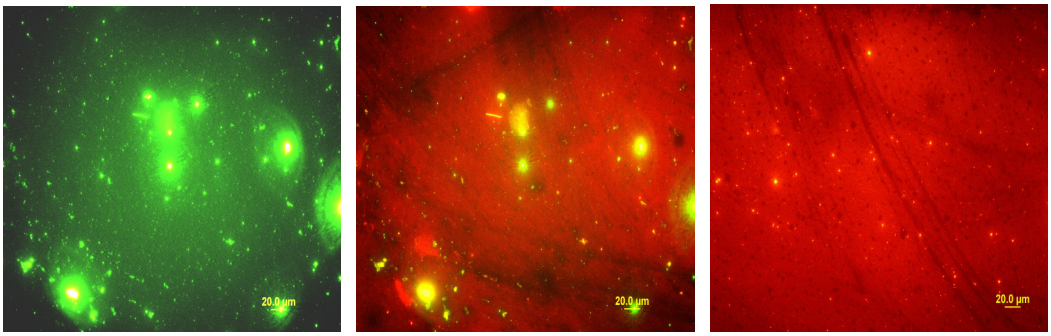
FT (Feed to thermophilic reactor) TE (Themophilic effluent)

FM (Feed to mesophilic reactor) ME (Mesophilic effluent)

Data from mean of two replicate sampling (± standard deviation)

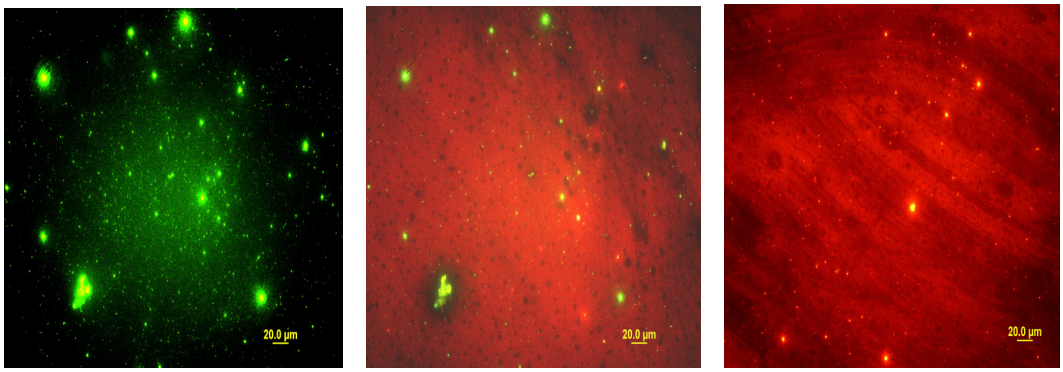
The microbial results show that while the overall numbers of viable anaerobic microorganisms in the themophilic and mesophilic reactor effluents were 2-fold more than the piggery influents, the *bacteria* number was significantly greater in the thermophilic effluent compared to the thermophilic influent and mesophilic effluent. The higher thermophilic bacteria number corresponded with the higher net extent of hydrolysis in the thermophilic anaerobic reactor (Table 6.6). In the case of the mesophilic microoganisms, while the effluent *archaea* number was found to be greater than the influent *archaea*, surprisingly they were also greater in number than the mesophilic *bacteria* and thermophilic *archaea*. As the *bacteria* domain include fermentative bacteria (hydrolytic and acidogenic) which have the fastest growth rates or lowest doubling times of 30 minutes among the anaerobic microorganisms, one would expect the mesophilic *bacteria* to out-number the mesophilic *archaea* at short HRT. However, hydrogen-utilising methanogens are also fast-growers like the acidogens and homoacetogens, with minimum doubling time of between 4 to 11 hours (Zhang and Noike, 1991). Thus, they rapidly grow in both the acidogenic and methane reactors producing methane and carbon dioxide (Gonenc and Keresteciogl u, 1990). Other possible factors that could have contributed to the higher mesophilic *archaea* result in particular, are described in section 5.4 (Chapter 5). Figures 6.4 and 6.5 show some selected fluorescent images of the *archaea* (a), *archaea* plus low-GC bacteria (b) and total bacteria (c) in the thermophilic and mesophilic effluents respectively.

(a) (b) (c)



Figures 6.4 (a), (b) and (c). Fluorescent images of *archaea* (green), *archaea* (green) plus low-GC bacteria (red) and total *bacteria* (red) in thermophilic reactor effluent respectively

(a) (b) (c)



Figures 6.5 (a), (b) and (c). Fluorescent images of *archaea* (green), *archaea* (green) plus low-GC bacteria (red) and total *bacteria* (red) in mesophilic reactor effluent respectively

T-RFLP molecular profiles generated using *archaeal* primers revealed the predominant methanogens present in the thermophilic effluent were closely related to the acetoclastic *Methanosarcina thermophila* while the mesophilic effluent contained more diverse methanogenic groups with hydrogenotrophic *Methanculleus* spp. and aceticlastic *Methanosarcina acetivorans* being the largest two groups (Table 6.8).

Table 6.8. T-RFLP molecular profile results of low-strength piggery influent and digested effluents from thermophilic (T) and mesophilic (M) anaerobic reactors at 2-d HRT

Piggery wastewater	*Methanoculleus* spp. (%)	*Methanosarcina acetivorans* (%)	*Methanosarcina thermophila* (%)	Others
T-influent	60	25	5	10
T-effluent			95	5
M-influent	60	25	5	10
M-effluent	40-95	40	10	0-05

Data are mean values (± standard deviation)

The proportions of methanogen populations of the thermophilic and mesophilic reactor effluents were noted to be different from those in the piggery influent which had hydrogenotrophic *Methanoculleus spp.* as the dominant group. Its broad methanogenic diversity was similar to that of the mesophilic reactor effluent.

To identify and quantify the phylogenetic microbial types within the bacteria domain, group-specific oligonucleotide FISH probes targeting alpha-, beta- and gamma-proteobacteria as well as the low- and high-GC bacteria were used. The abundance of each specific group of bacteria was expressed as percentage of the total bacteria domain. Figure 6.6 illustrates the distribution of various phylogenetic groups of bacteria.

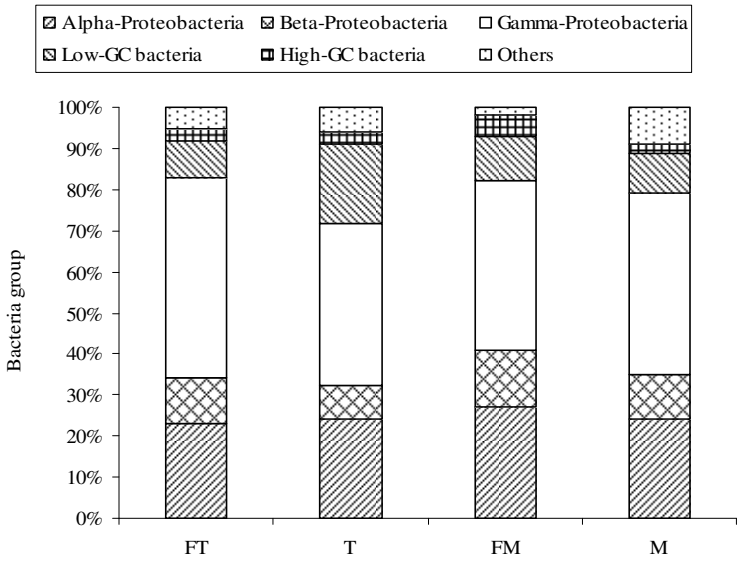


Figure 6.6. Comparison of the distribution of phylogenetic groups of *bacteria* in the influents (FT, FM) and effluents of the thermophilic (T) and mesophilic (M) first-stage anaerobic reactors at 2-d HRT using 16S rRNA-group specific FISH probes

The *bacteria* present in the piggery influent contained largely gamma-proteobacteria, a group that includes pathogenic *E. coli*, *Enterobacter*, *Salmonella*, followed by alpha-proteobacteria, beta-proteobacteria, low-GC bacteria, high-GC bacteria and others in decreasing proportions. Although gamma-proteobacteria still remained the largest group in thermophilic and mesophilic effluents, there was some 10% reduction in this group in the thermophilic effluent whereas there was no reduction being observed in the mesophilic effluent. The low-GC bacteria group which includes the heat-resistant spore-forming, hydrogen-producing *Bacillus* and *Clostridium* genera showed an increase of 10% in the thermophilic effluent. Greater

proportion of other unidentified group was found in the mesophilic effluent than in the thermophilic effluent and the influents. Appendices 1 and 2 list the bacteria genera that belong to the phylogenetic groups of Gram-negative proteobacteria and Gram-positive G+C bacteria respectively.

6.3.1.2. Performance comparison of thermophilic (55°C) and mesophilic (37°C) first-stage anaerobic reactors treating low-strength piggery wastewaters with synthetic complex wastewaters at 2-day hydraulic retention time

Table 6.9 presents some selected key chemical data of the piggery and synthetic organic wastewaters for comparison purposes. Although total alkalinity of the synthetic wastewater was not determined, its substantially lower ammonia-nitrogen concentration (ammonium-nitrogen and free ammonia) and the rapid acidification of its influent soluble organic fraction in conjunction with a rapid pH drop from its artificially elevated pH of 8 to pH around 4 during anaerobic treatment clearly indicated that the synthetic wastewater lacked natural buffering capacity against VFA souring.

Table 6.9. Comparison of some key chemical characteristics of the piggery wastewater and synthetic complex organic wastewater

Piggery wastewater	pH	Total alkalinity (mg CaCO3/L)	Soluble COD (mg/L)	Total VFA (mg COD/L)	NH_4^+-N (mg/L)	Free NH_3-N (mg/L)
T-influent	7.6 (0.1)	2190 (170)	2697 (139)	2893 (96)	690 (99)	3 (1)
T-effluent	7.7 (0.2)	2505 (64)	2750 (122)	2209 (187)	690 (61)	105 (27)
M-Influent	7.5 (0)	2100 (297)	2542 (61)	2438 (272)	655 (7)	2 (0)
M-effluent	7.6 (0.1)	2475 (106)	2631 (99)	2132 (92)	650 (46)	31 (11)
Synthetic wastewater						
T-influent	8.0 (0.1)	na	750 (24)	0	1.3 (0)	0.015
T-effluent	4.4 (0.0)	na	1145 (54)	874 (91)	10 (1)	0.001
M-Influent	8.0 (0.1)	na	744 (77)	0	1.3 (0)	0.015
M-effluent	3.9 (0.1)	na	1790 (88)	2023 (219)	0.4 (0.2)	0.000

Data are mean values (± standard deviation) na (not analysed)

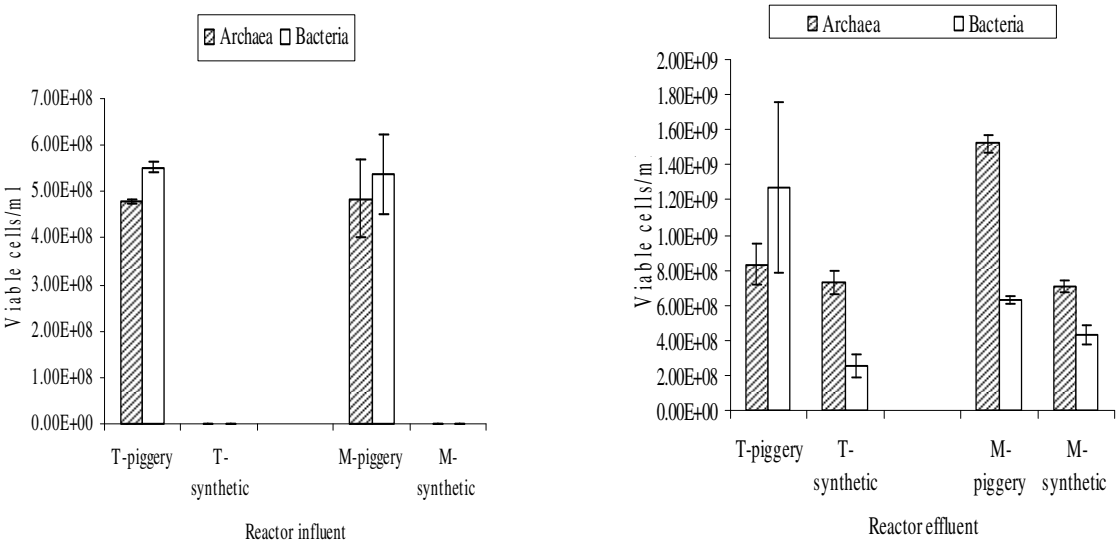
T (thermophilic) M (mesophilic)

On the other hand, the piggery wastewater had substantially higher ammonium-nitrogen concentration than the synthetic wastewater and a slightly alkaline pH that

resisted souring by the high influent VFA concentration. Its high natural buffering capacity was reflected by the high total alkalinity measured.

Besides the observed differences in their chemical characteristics, differences in their biological characteristics were also noted. As shown in Figure 6.7 (a), while piggery influent fed to the reactor contained high levels of anaerobic *archaea* and *bacteria*, there were no detectable viable cells apart from some large auto-fluorescent non-cell particles in the synthetic influent. The lack of anaerobic microorganisms in the synthetic wastewater was not surprising given that it was prepared from commercial pig food pellets in distilled water. In addition, it was not inoculated or seeded with sludge containing anaerobic microorganisms.

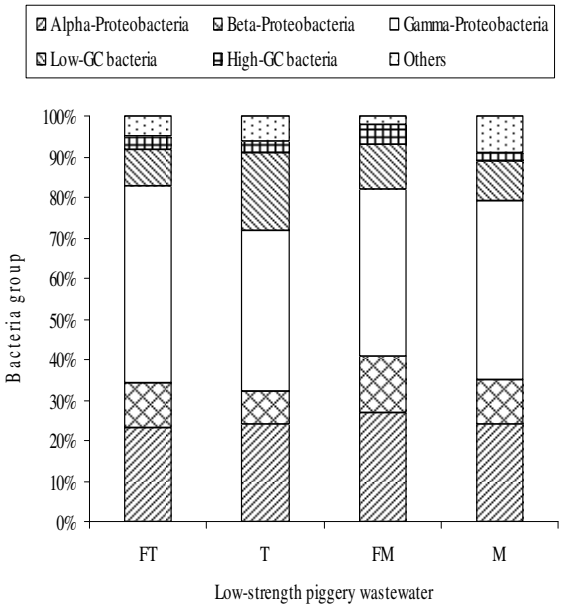
(a) (b)



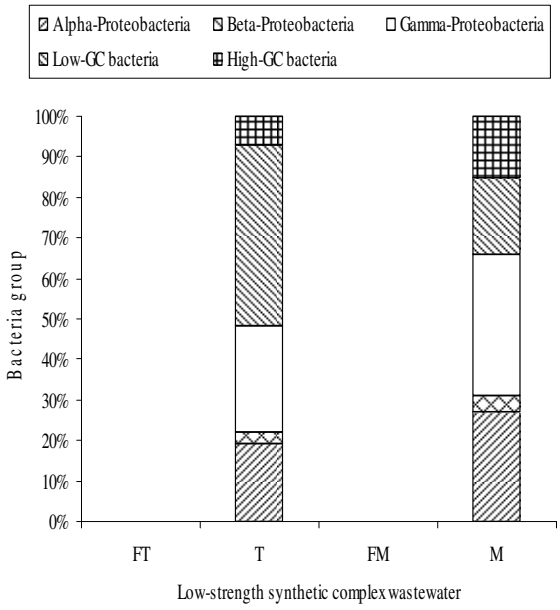
Figures 6.7 (a) and (b). Comparison of anaerobic *archaea* and *bacteria* populations in the low-strength thermophilic (T) and mesophilic (M) piggery and synthetic influents as well as reactor effluents (error bars indicate standard deviations)

Both thermophilic and mesophilic anaerobic reactors fed with piggery wastewaters had higher number of anaerobic *archaea* and *bacteria* in the effluents compared to their counterpart reactors fed with synthetic wastewaters (Figure 6.7 (b)). Figures 6.8 (a) and (b) illustrate the differences in the proportions of anaerobic bacteria groups found in the piggery wastewater and synthetic wastewater respectively.

(a)



(b)



Figures 6.8 (a) and (b). Comparison of anaerobic *bacteria* groups in the low-strength thermophilic (T) and mesophilic (M) piggery and synthetic influents (FT, FM) as well as reactor effluents (T, M) respectrively

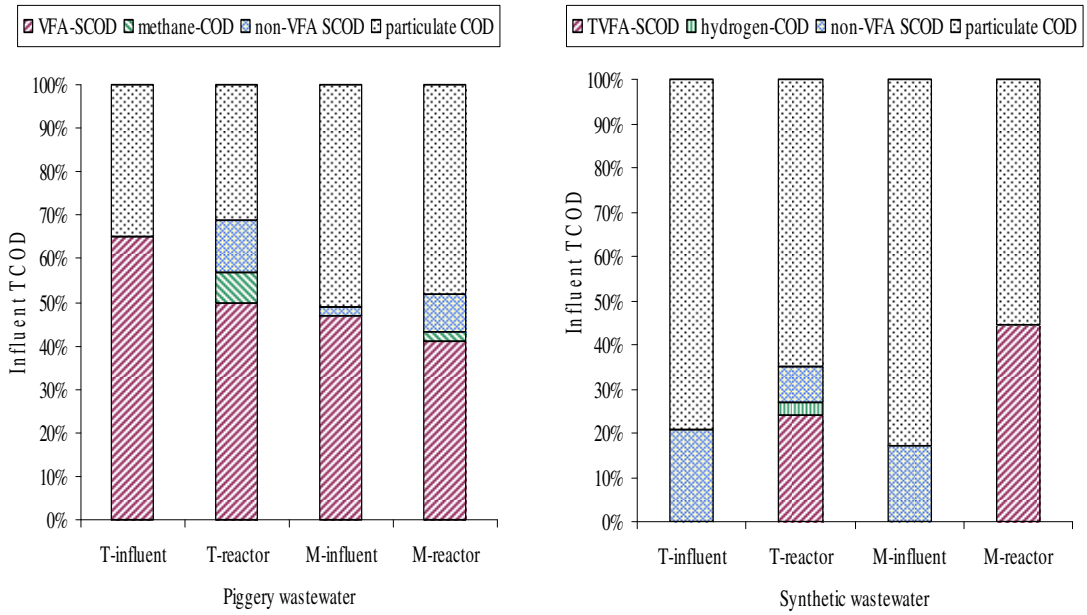
While gamma-proteobacteria was the largest group in the thermophilic and mesophilic piggery effluents as well in the mesophilic synthetic effluent, low-GC bacteria was the largest group in the thermophilic synthetic effluent. About 10% decrease in gamma-proteobacteria and 10% increase in low-GC bacteria were observed with the piggery effluent after treatment in the thermophilic anaerobic reactor. No similar trends were observed with these two phylogenetic groups after mesophilic reactor treatment of the piggery wastewater. Piggery effluents were noted to have greater bacteria diversity than the synthetic effluents.

The detection of viable anaerobic microorganisms, particularly the active *bacteria* in the thermophilic and mesophilic anaerobic reactors treating synthetic wastewaters showed that these microorganisms introduced by the municipal digested sludge used to seed the reactor at the start, were responsible for the increased solubilisation of particulate organic matter and conversion of the soluble organic matter to volatile fatty acids (Table 6.9). While methane was not detected in the biogas apart from small amount of hydrogen from the reactors treating synthetic wastewater, the detection of fluorescent *archaea* cells in both the thermophilic and mesophilic reactor effluents suggested that the *archaea* introduced by the sludge were still viable

but severely inhibited by the low pH from consuming the methanogenic substrates such as acetate and hydrogen.

Figures 6.9 (a) and (b) present graphical comparisons of the influents and effluents COD material balance of the anaerobic reactors treating piggery and synthetic wastewaters respectively.

(a) (b)



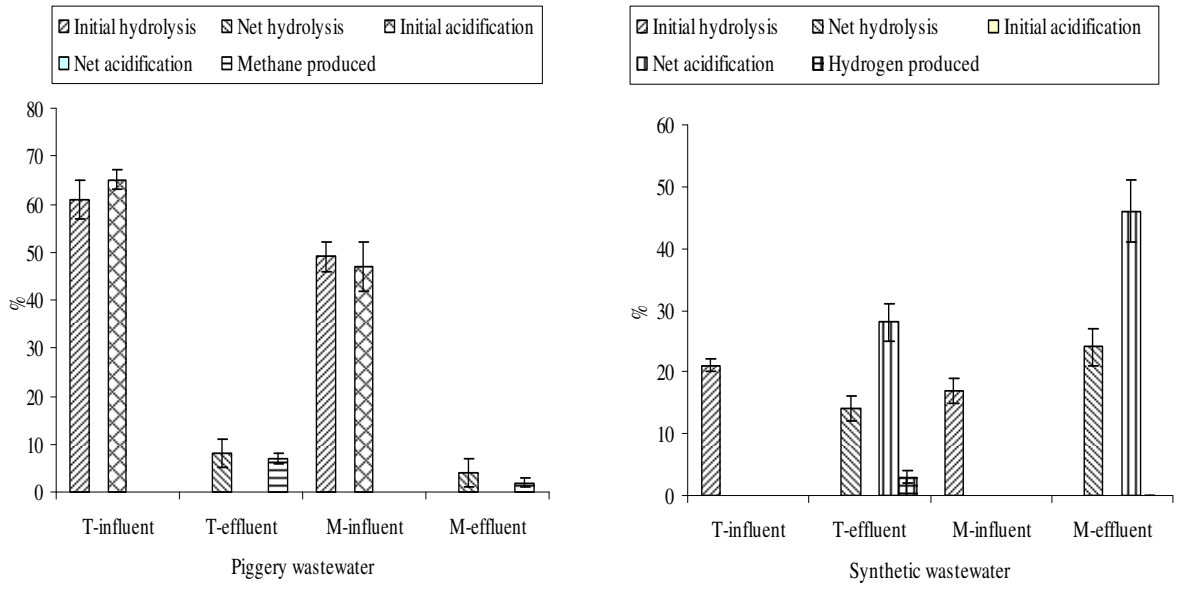
Figures 6.9 (a) and (b). COD material balance of low-strength thermophilic (T) and mesophilic (M) piggery and synthetic influents as well as reactor effluents respectrively

The two types of wastewater clearly differed in their proportions of particulate and soluble organics. While the piggery wastewater had lower particulate organics (35 to 51%) and higher soluble organics (47 to 65%) which had been almost completely acidified to VFAs as a result of prior digestion in the pig rumen and in the piggery holding sump, the synthetic wastewater contained much higher proportions of particulate organic matter (79-83%) and lesser soluble organic matter (17-21%). The latter was not in acidified form as the synthetic wastewater lacked natural anaerobic microflora to ferment it (Figure 6.8). During thermophilic and mesophilic anaerobic treatment, the piggery wastewater influent underwent acetogenesis and methanogenesis whereby some of the VFAs in the piggery wastewater influent were converted to methane plus carbon dioxide and other unidentified soluble organic components (non-VFA SCOD). In contrast, the synthetic wastewater influent

underwent hydrolysis and acidogenesis whereby small amount of particulate organics was solubilised while the soluble organics was completely acidified to VFAs plus hydrogen, the latter only observed during thermophilic digestion.

Figures 6.10 (a) and (b) graphically compare the organics conversion efficiency of the thermophilic and mesophilic anaerobic reactors treating piggery and synthetic wastewaters respectively.

(a) (b)



Figures 6.10 (a) and (b). Comparison of the extent of initial hydrolysis, net hydrolysis, initial acidification, net acidification, methane production and hydrogen production of the thermophilic (T) and mesophilic (M) first-stage naerobic reactors treating piggery wastewaters and synthetic complex wastewater respectively (error bars indicate standard deviations)

Both thermophilic and mesophilic anaerobic treatment of the synthetic wastewater (Figure 6.10 (b)) produced significantly ($p<0.05$) higher net hydrolysis of the particulate organics and net acidification of the soluble organics compared to the piggery wastewater (Figure 6.10 (a)). While mesophilic anaerobic reactor treating synthetic wastewater produced significantly higher organics hydrolysis and acidification than the thermophilic anaerobic reactor, the reverse order was observed with anaerobic treatment of the piggery wastewater.

6.3.2. Semi-continuous first-stage thermophilic (55^{o}C) CSTR anaerobic reactors treating medium- and high-strength piggery wastewaters with and without pH reduction at 2-day HRT

6.3.2.1. Piggery wastewater without pH reduction

For comparison purposes, data from the earlier first-stage thermophilic reactor experiment on the diluted low-strength piggery wastewater (section 6.3.1.1) were included in all the Tables and Figures below alongside the data of medium- and high-strength piggery wastewaters at 2-day HRT.

pH, alkalinity, ammonium-nitrogen, free ammonia, soluble phosphorus and sulphate

As shown in Table 6.10, despite increasing the organic strength of the piggery influent from 4 g/L (low) to 7 g/L (mid) to 13 g/L (high) TCOD, the effluent pH showed no signs of dropping at 2-d HRT.

Table 6.10. pH, total alkalinity, ammonium-nitrogen, free ammonia, soluble phosphorus and sulphate concentrations of the low-, middle- and high-strength influents and effluents at 2-d HRT

Thermophilic piggery wastewater	pH	Total alkalinity (mg $CaCO_3$/L)	NH_4^+-N (mg N/L)	Free NH_3-N (mg N/L)	Soluble phosphorus (mg P/L)	Sulphate (mg SO_4^{2-}/L)
Influent (low)	7.6 (0.1)	2190 (170)	690 (99)	3 (1)	46 (1)	na
Effluent (low)	7.7 (0.2)	2505 (64)	690 (61)	105 (27)	40 (6)	na
Influent (mid)	7.4 (0.1)	3600 (0)	985 (7)	3 (1)	60 (2)	na
Effluent (mid)	8.2 (0)	4010 (121)	968 (28)	355 (33)	32 (4)	na
Influent (high)	7.3 (0.1)	5595 (488)	1800 (0)	4 (1)	112 (26)	1200
Effluent (high)	8.1 (0.1)	6500 (165)	2150 (191)	788 (85)	34 (5)	590

Data are mean values (± standard deviation)

Instead, effluent pH crept up by a unit from around 7 to around 8 in response to the increase in total alkalinity and ammonia-nitrogen concentrations due to increasing influent TCOD concentration. While the effluent free ammonia concentration exhibited increasing trend with increasing organic strength, soluble phosphorus concentration in contrast showed a decreasing trend. More than 50% of the high-strength influent sulphate concentration was also reduced in the effluent.

Total and volatile suspended solids (TSS and VSS)

The high VSS:TSS ratio of the undiluted piggery wastewater in Table 6.11 concurred with the low-strength influent that the suspended solids were composed almost entirely of organic materials.

Table 6.11. Total and volatile suspended solids in the low- and high- strength influents and effluents at 2-d HRT

Thermophilic piggery wastewater	TSS (g/L)	VSS (g/L)	VSS removal (%)	VSS/TSS
Influent (low)	1.7 (0.2)	1.6 (0.2)		98 (12)
Effluent (low)	1.5 (0.5)	1.2 (0.3)	33 (14)	
Influent (mid)	na	na		
Effluent (mid)	na	na		
Influent (high)	3.5 (0.8)	3.2 (0.7)		92 (4)
Effluent (high)	2.7 (0.5)	2.3 (0.6)	28 (14)	

Data are mean values (± standard deviation) na (not available)

Statistical t-test comparison of the VSS removal data of the low and high-strength effluents showed there was no significant difference ($p>0.05$) between them. The results mirrored the calculated net extent of hydrolysis data given in Table 6.15.

COD, volatile fatty acids and biogas

Table 6.12 gives the COD (total and soluble) and total VFA-COD data of the medium- and high-strength piggery influents and thermophilic reactor effluents. For comparison purposes, data of the low-strength piggery influent and effluent from the earlier thermophilic first-phase reactor experiment were also included in the table. The percentage ratio of soluble COD/total COD was calculated to determine the amount of organics that had solubilised in the piggery influent as received. Similarly, the percentage ratio of total VFA-COD/soluble COD was calculated to determine the amount of soluble organics that had acidified to volatile fatty acids in the piggery influent as received.

Table 6.12. Chemical oxygen demand (total and soluble) and total VFA concentrations of low-, medium- and high-strength piggery influents and thermophilic digested effluents at 2-d HRT

Thermophilic piggery wastewater	Total COD (mg/L)	Soluble COD (mg/L)	Total VFA (mg COD/L)	SCOD/TCOD	TVFA/SCOD
Influent (low)	4449 (211)	2697 (139)	2893 (96)	61 (3)	107 (4)
Effluent (low)	4384 (404)	2750 (122)	2209 (187)		
Influent (mid)	7175 (485)	5182 (290)	5118 (114)	72 (3)	99 (5)
Effluent (mid)	5054 (448)	3651 (227)	2551 (192)		
Influent (high)	12865 (774)	7619 (747)	8139 (340)	59 (3)	109 (13)
Effluent (high)	10365 (766)	7122 (422)	6009 (314)		

Data are mean values (± standard deviation)

The high SCOD:TCOD ratios of the mid- and high-strength (undiluted) piggery wastewaters concurred with the earlier observation that more than half of the organic matter in the piggery wastewater as received was already in soluble form. Their near 100% of TVFA:SCOD ratios also concurred with earlier observation that the soluble organic matter had completely acidified to VFAs by the indigenous acidogenic microbial populations prior to the reactor experiments.

The amount of COD (total and soluble) and TVFA-COD removed from the low-, mid- and high-strength piggery influents after thermophilic anaerobic treatment at 2-day HRT is graphically presented in Figure 6.11. Highest organics removal was observed to occur in the medium-strength effluent while comparable TVFA removals were observed between the low- and high-strength effluents.

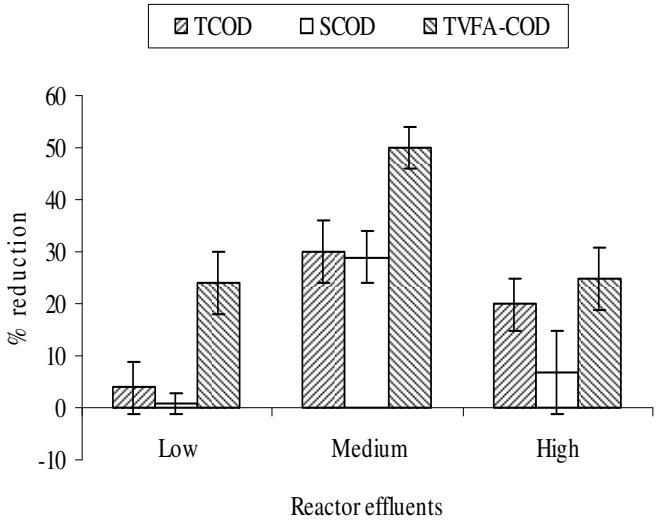


Figure 6.11. COD (total and soluble) and total VFA reductions of thermophilic digested effluents at 2-d HRT (error bars indicate standard deviations)

As shown in Table 6.13 and Figure 6.12, significantly ($p<0.05$) more acetate, n-butyrate, i-and n-valerate were degraded in the medium-strength piggery influent than either the low- or high-strength influent. More i-butyrate and n-valerate were degraded in the high-strength effluent than the low-strength effluent.

Table 6.13. Volatile fatty acid concentrations of the low-, medium- and high-strength piggery influents and thermophilic digested effluents at 2-d HRT

Thermophilic piggery wastewater	Acetate (mg COD/L)	Propionate (mg COD/L)	i-butyrate (mg COD/L)	n-butyrate (mg COD/L)	i-valerate (mg COD/L)	n-valerate (mg COD/L)	Caproate (mg COD/L)
Influent (low)	1086 (46)	639 (70)	127 (19)	437 (12)	292 (7)	234 (25)	78 (4)
Effluent (low)	741 (89)	474 (59)	158 (17)	243 (119)	335 (16)	170 (28)	88 (7)
Influent (mid)	2156 (53)	1049 (52)	229 (41)	623 (12)	487 (19)	445 (39)	129 (2)
Effluent (mid)	1003 (113)	1074 (91)	14 (30)	69 (9)	214 (22)	168 (15)	10 (10)
Influent (high)	3619 (191)	1916 (111)	325 (45)	701 (19)	801 (31)	586 (77)	192 (4)
Effluent (high)	2652 (141)	1964 (169)	81 (91)	223 (19)	687 (25)	327 (19)	75 (88)

Data are mean values (± standard deviation)

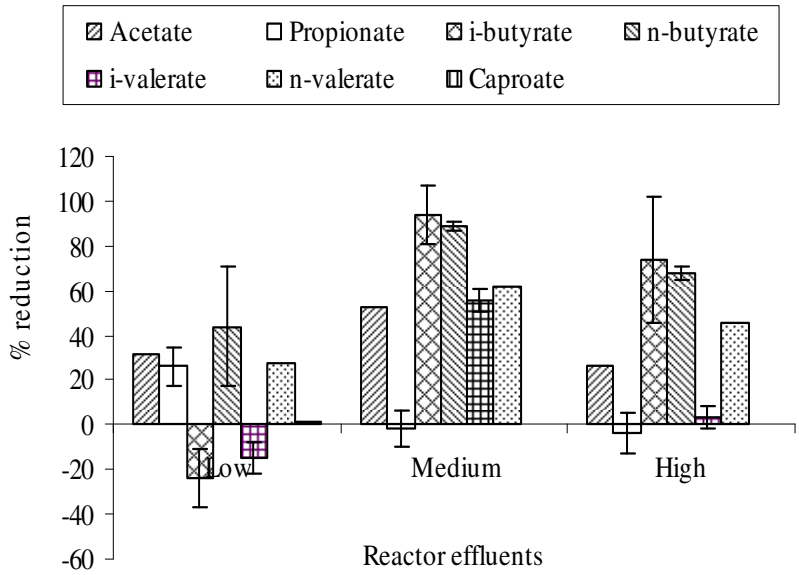


Figure 6.12. Percentage removal of VFA-COD in the thermophilic digested effluents at 2-d HRT (error bars indicate standard deviations)

Table 6.14 showed the reactor treating medium-strength piggery influent produced the highest ($p<0.05$) specific methane yield compared to the reactors treating low- and undiluted high-strength influent.

Table 6.14. Biogas composition and specific methane yields from the thermophilic first-stage reactors treating low-, medium- and high-strength piggery wastewaters

Feed TCOD strength	OLR (g TCOD fed /L/d)	Total VFA removed rate (g- SCOD/L/d)	% methane	% carbon dioxide	Actual specific methane yield (L CH_4/g- SCOD removed) at stp
Low	2.225 (0.080)	0.329 (0.098)	65 (3)	24 (2)	0.154 (0.033)
Mid	3.507 (0.079)	1.284 (0.096)	84 (2)	20 (1)	0.292 (0.039)
High	6.433 (0.439)	1.024 (0.224)	72 (1)	30 (2)	0.229 (0.037)

Data are mean values of 8 measurements (± standard deviation)

It is noted that the methane content of the biogas from the reactor treating medium-strength influent was rather high which could be due to dissolution of some carbon dioxide in the effluent which elevated its concentration (Clarke, personal communication). The actual specific methane yields of the medium- and high-

strength piggery effluents were noted to be significantly lower than the theoretical value of 0.35 L/g COD removed at standard conditions.

COD material balance

Figure 6.13 shows the COD material balance constructed from the various COD components (SCOD, VFA-COD and methane-COD) present in the low-, medium- and high-strength pH-unadjusted influents and effluents relative to their respective influent TCOD concentrations.

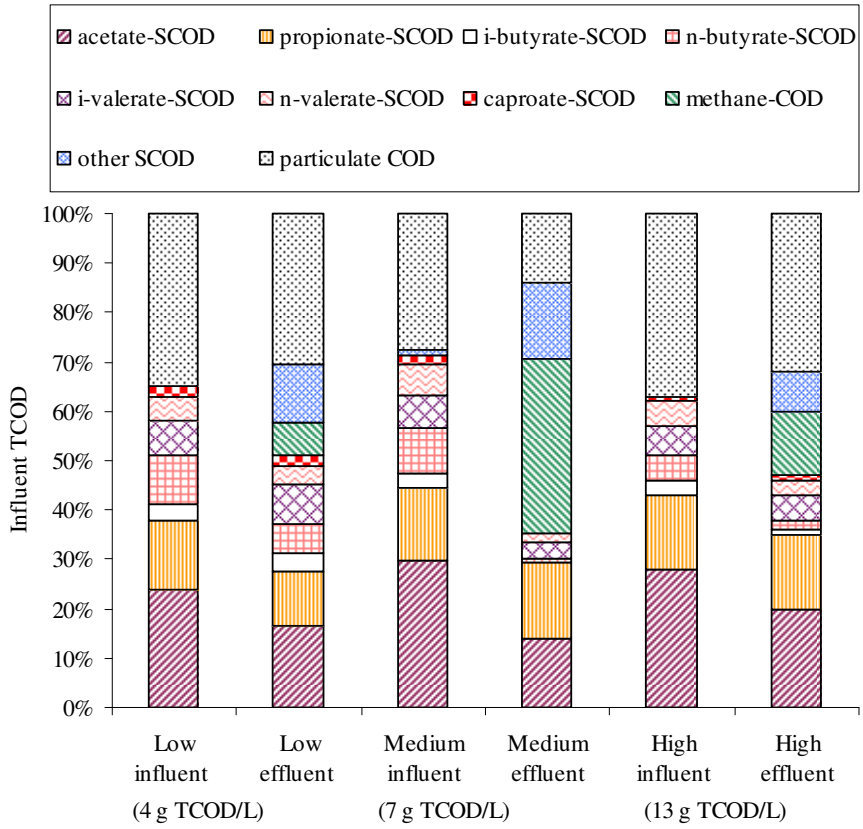


Figure 6.13. COD material balance of the low-, medium- and high-strength piggery influents and thermophilic digested effluents

Diluting the high-strength piggery wastewater to mid-strength (7 g/L TCOD) was observed to result in highest amount of the influent TVFA being lost compared to the low- and undiluted high-strength wastewaters. Methane production still occurred at the short HRT of 2-day irrespective of their organic strength, suggesting that the methanogenic microorganisms could not be completely inhibited. Non-VFA soluble organic fractions (other SCOD) in the effluents were noted to be higher than the influents following digestion in the thermophilic reactors. Overall, the COD material

balance of the thermophilic effluents show the proportions of un-degraded COD ranged from 14 to 32% as particulate COD and 51 to 62% as soluble organics which were comprised of VFAs, non-VFAs components and methane biogas.

Reactor performance in the conversion of organics

Data on the organics conversion of the low, mid and high organic strength piggery wastewaters (Table 6.15) show small increases in net hydrolysis of the particulate organics while negligible to hardly any additional acidification of the dissolved organic matter took place in the thermophilic reactors at 2-day HRT.

Table 6.15. Comparison of the degree of net hydrolysis, net acidification and methanogenesis in the thermophilic first-stage anaerobic reactors treating low-, medium- and high-strength piggery wastewaters

Sample Piggery water strength Unit	Effluent Low % feed TCOD	Effluent Medium % feed TCOD	Effluent High % feed TCOD
Extent of net hydrolysis	8 (3)	14 (4)	10 (5)
Extent of net acidification	0	2 (3)	0
Extent of methanogenesis	7 (1)	35 (2)	13 (2)

Data are mean values (± standard deviation)

Statistical t-test analysis ($p<0.05$) of the organics conversion data showed anaerobic treatment of medium-strength piggery wastewater produced the highest net hydrolysis, acidification and methanogenesis compared to the low- and high-strength piggery wastewaters. While net hydrolysis was comparable between the low- and high-strength piggery wastewaters, the latter produced significantly higher methane yield than the low-strength piggery wastewater.

Anaerobic microbial populations

Tables 6.16 and 6.17 give the bacteria and methanogen populations enumerated by molecular FISH and real-time PCR methods respectively in the low-, medium- and high-strength piggery influents and digested effluents.

Table 6.16. Quantification of viable bacteria and methanogen populations in the low-, medium- and high-strength pH-unadjusted piggery influents and thermophilic digested effluents using 16S rRNA-specific FISH probes of EUBMIX and ARC-915 respectively

Piggery	Bacteria (counts/ml)	Methanogens (counts/ml)
Influent (low)	$(5.52 \pm 0.11) \times 10^8$	$(4.79 \pm 0.04) \times 10^8$
Effluent (low)	$(1.27 \pm 0.49) \times 10^9$	$(8.31 \pm 1.17) \times 10^8$
Influent (mid)	$(6.04 \pm 1.14) \times 10^8$	$(1.21 \pm 0.21) \times 10^9$
Effluent (mid)	$(3.03 \pm 0.55) \times 10^8$	$(1.17 \pm 0.17) \times 10^9$
Influent (high)	$(8.70 \pm 0.07) \times 10^8$	$(5.90 \pm 1.49) \times 10^8$
Effluent (high)	$(1.25 \pm 0.10) \times 10^9$	$(8.19 \pm 0.54) \times 10^8$

Data are mean values of replicate measurements (± standard deviation)

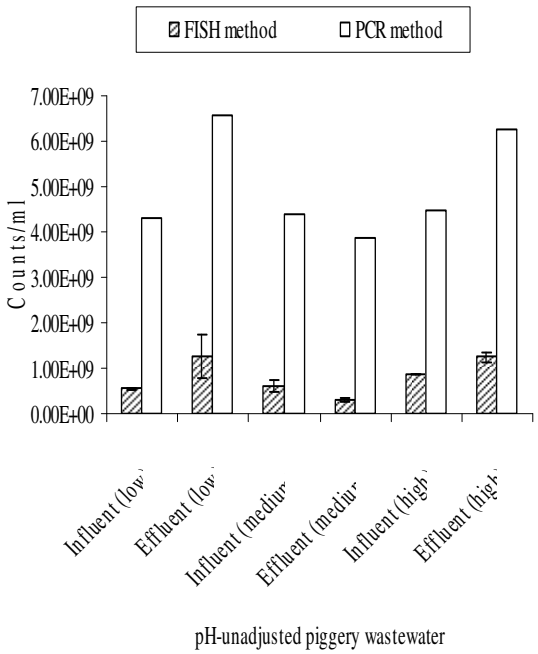
The numbers of bacteria and methanogens counted by FISH method in the low- and high-strength digested effluents show significant increase over their influents. In contrast, the number of *bacteria* in the medium-strength digested effluent was significantly lower than the influent whereas the number of methanogens remained relatively constant. It is surprising to note the significantly higher methanogen number in the medium-strength influent compared to the high-strength influent (Table 6.16) as well as the significantly lower bacteria number in the medium-strength digested effluent compared to its low-strength counterpart.

Table 6.17. Bacteria and methanogen populations estimated by real-time PCR method in the low-, medium- and high-strength pH-unadjusted piggery influents and thermophilic digested effluents

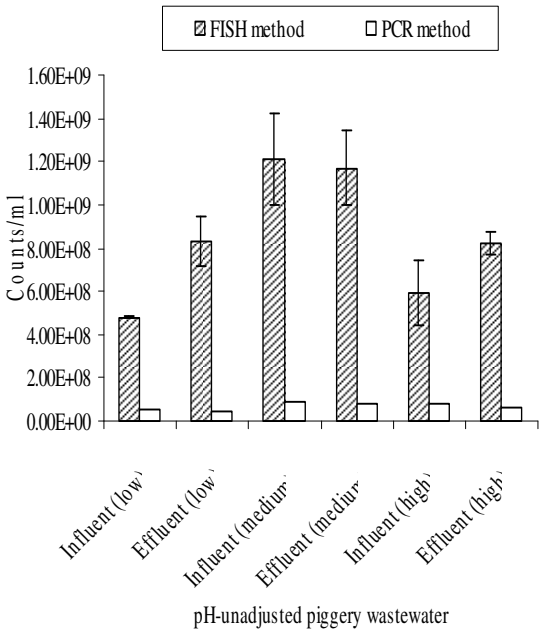
pH-unadjusted piggery	Bacteria (counts/ml)	Methanogens (counts/ml)
Influent (low)	4.30×10^9	5.73×10^7
Effluent (low)	6.55×10^9	4.65×10^7
Influent (medium)	4.37×10^9	8.61×10^7
Effluent (medium)	3.85×10^9	8.17×10^7
Influent (high)	4.49×10^9	7.69×10^7
Effluent (high)	6.27×10^9	6.54×10^7

Graphical presentations of the *bacteria* and methanogen counts by FISH and real-time PCR methods in Figures 6.14 (a) and (b) respectively revealed huge data discrepancies exist between the two molecular methods.

(a) Bacteria



(b) Methanogens



Figures 6.14 (a) and (b). Comparisons of *bacteria* and methanogen numbers by molecular FISH and real-time PCR methods respectively (error bars indicate standard deviations)

As expected, the bacteria counts by PCR method were consistently higher than the cell counts by FISH method by 1 to 2 orders of magnitude due to its inability to exclude the DNA of non-viable cells. Surprisingly, the PCR methanogen counts were much lower than the cell counts by FISH method by the same orders of magnitude as the bacteria counts. It appears that an inherent systemic bias was introduced either in the real-time PCR or FISH molecular method for the quantification of methanogenic *archaea* population. Despite the huge differences in microbial counts between the two molecular methods, the bacteria profiles of the influents and effluents by PCR method were similar to the FISH profiles as illustrated in Figure 6.14 (a).

Methanogen profiles of the medium-strength influent and effluent by PCR method also appear to be consistent with the *archaea* profiles obtained by FISH method. Figure 6.15 shows the methanogen profile to be similar to that of the specific methane yield, with the medium-strength piggery effluent containing highest number of methanogens and highest specific methane yield.

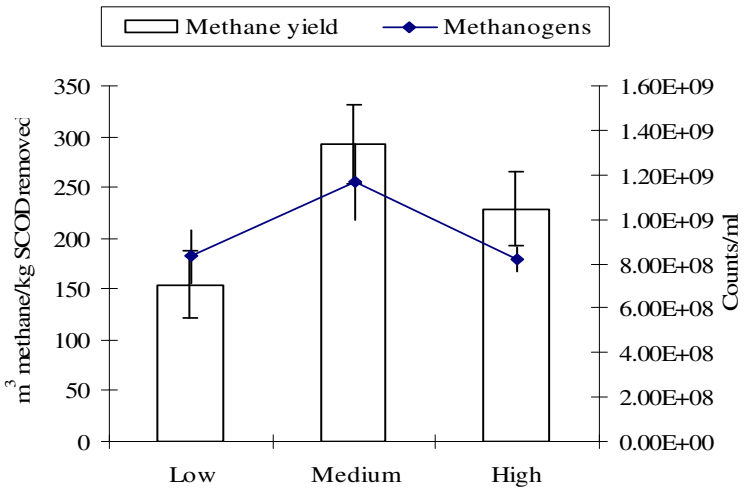
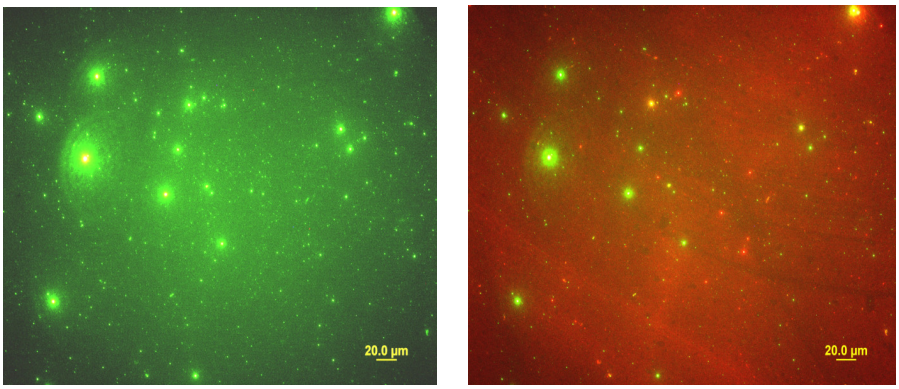


Figure 6.15. Methanogen (*archaea*) FISH counts and specific methane yields as a function of the effluent organic carbon concentrations (error bars indicate standard deviations)

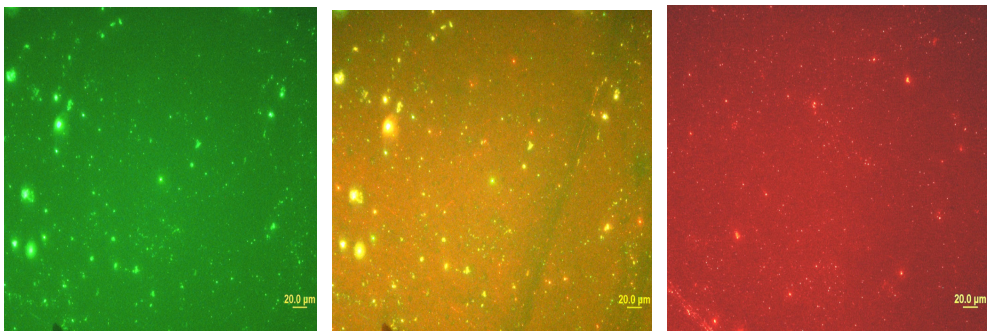
Figures 6.16 (a) and (b) show the fluorescent images of the *archaea* and total *bacteria* (hydrolytic, acidogenic and acetogenic bacteria) in the medium-strength effluent while Figure 6.17 (a), (b) and (c) show the *archaea*, *archaea* plus low-GC bacteria and total *bacteria* in the high-strength effluents respectively.

(a) (b)



Figures 6.16 (a) and (b). Fluorescent images of *archaea* (green) and total *bacteria* (red) in thermophilic reactor effluent (pH-unadjusted medium-strength) respectively

(a) (b) (c)



Figures 6.17 (a), (b) and (c). Fluorescent images of *archaea* (green), *archaea* (green) plus low-GC bacteria (red) and total *bacteria* (red) in thermophilic reactor effluent (pH-unadjusted high-strength) respectively

T-RFLP molecular profiles generated using *archaeal* primers revealed the predominant methanogens present in the diluted low- and medium-strength reactor influents were made up of largely hydrogenotrophic *Methanculleus* spp. with minor proportions of acetoclastic *Methanosarcina acetivorans* plus other unidentified *archaea* and a small proportion closely related to acetoclastic *Methanosarcina thermophila*. The latter population quickly became the dominant group in the thermophilic low- and medium-strength reactor effluents (Table 6.18).

Table 6.18. T-RFLP molecular profile results of pH-unadjusted piggery influents and thermophilic digested effluents at 2-d HRT

Piggery wastewater	*Methanoculleus* spp. (%)	*Methanosarcina acetivorans* (%)	*Methanosarcina thermophila* (%)	Others
Influent (low)	60	25	5	10
Effluent (low)			95	5
Influent (medium)	60	25	5	10
Effluent (medium)			90-95	5-10
Influent (high)	30	30	20	20
Effluent (high)	5		90-95	5

In the high-strength reactor influent, it was noted that lower proportion of hydrogenotrophic *Methanoculleus* spp. and higher proportions of the acetoclastic *archaea* were reported compared to the diluted low- and medium-strength influents. The lack of consistency with dilution could be due to analysis error. Despite the irregularity in *Methanoculleus* spp. estimate, acetoclastic *Methanosarcina thermophila* became the dominant group in the thermophilic high-strength reactor effluent.

To gain an insight into the phylogenetic groups of bacteria within the *bacteria* domain, samples from the high-strength piggery influent and digested effluent were selected for study. Group-specific oligonucleotide FISH probes targeting alpha-, beta- and gamma-proteobacteria as well as the low- and high-GC bacteria were used to identify and quantify them. The abundance of each specific group of bacteria was expressed as percentage of the total *bacteria* domain. Figure 6.18 illustrates the distribution of the various phylogenetic bacterial groups in the high-strength piggery wastewaters. For comparison purposes, bacteria distributions of the low-strength piggery influent and effluent were also included.

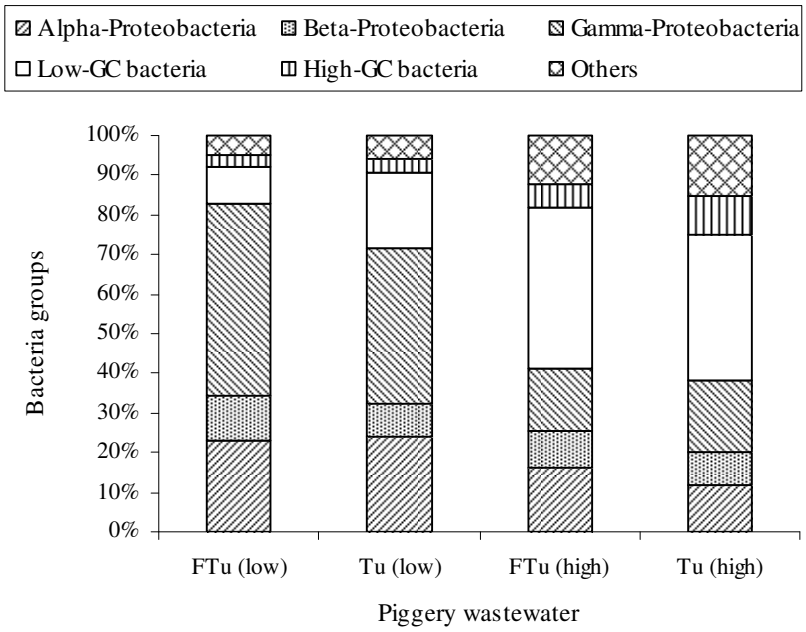


Figure 6.18. Comparison of the distribution of phylogenetic groups of *bacteria* in the influents (FTu) and effluents (Tu) of the thermophilic (T) low- and high-strength anaerobic reactors at 2-d HRT using 16S rRNA-group specific FISH probes

In contrast to the low-strength piggery wastewaters where gamma-proteobacteria formed the largest group (39-48%), the largest group of *bacteria* present in the high-strength piggery influent and effluent was the low-GC bacteria (37-40%), a group that includes the spore-forming, hydrogen-producing *Bacillus* and pathogenic *Clostridium* genera. It appeared that there was no reduction in the proportion of gamma-proteobacteria in the high-strength effluent unlike in the low-strength effluent. Similarly, no reduction in the low-GC bacteria group was observed in the high-strength effluent whereas this group showed an increase in the low-strngth

effluent. The proportion of other unidentified group was greater in the high-strength piggery wastewaters than their low-strength counterparts. Appendices 1 and 2 list the bacteria genera that belong to the phylogenetic groups of Gram-negative proteobacteria and Gram-positive G+C bacteria respectively.

6.3.2.2. Piggery wastewater with pH reduction to 5.5

pH, total alkalinity, ammonium-nitrogen, free ammonia and soluble phosphorus

As shown in Table 6.19, reducing the influent pH from around 7.4 to 5.5 had the effect of maintaining the free ammonia at a negligible concentration in the reactor effluent. In contrast to the pH-unadjusted piggery influents, soluble phosphorus concentrations remained unchanged after reactor treatments.

Table 6.19. pH, total alkalinity, ammonium-nitrogen and free ammonia concentrations of the medium- and high-strength influents with pH-reduction and thermophilic digested effluents at 2-d HRT

Thermophilic piggery wastewater	pH	Total alkalinity (mg $CaCO_3$/L)	NH_4^+-N (mg/L)	Free NH_3-N (mg/L)	Soluble phosphorus (mg/L)
Influent (medium)	5.4 (0.1)	1140	1050 (71)	0	77 (14)
Effluent (medium)	5.4 (0.2)	1025 (74)	988 (25)	1 (0)	75 (8)
Influent (high)	5.4 (0.1)	1922 (233)	2000 (0)	0	180 (0)
Effluent (high)	5.7 (0.1)	1965 (57)	2050 (71)	4 (1)	180 (0)

Data are mean values (± standard deviation)

Total and volatile suspended solids (TSS and VSS)

While there were no statistically significant differences in the TSS and VSS concentrations between the mid-strength piggery influent and effluent, significant differences were observed between the high-strength influent and effluent (Table 6.20).

Table 6.20. Total and volatile suspended solids in the medium- and high-strength influents with pH-reduction and thermophilic digested effluents at 2-d HRT

Thermophilic piggery wastewater	TSS (g/L)	VSS (g/L)	VSS removal (%)
Influent (medium)	1.0 (0.2)	1.1 (0.4)	
Effluent (medium)	1.4 (0.4)	1.2 (0.3)	3 (5)
Influent (high)	3.4 (0.8)	3.4 (0.8)	
Effluent (high)	2.7 (0.5)	2.6 (0.6)	25 (11)

Data are mean values (± standard deviation)

The increased VSS reduction in the high-strength piggery influent indicated improved hydrolysis and acidification of the organic matter.

COD, volatile fatty acids and biogas

Table 6.21 gives the COD (total and soluble) and total VFA data of the medium- and high-strength pH-reduced piggery influents and effluents of the thermophilic first-phase anaerobic reactors. Table 6.22 and Figure 6.19 show the concentrations and percentage reductions of volatile fatty acids.

Table 6.21. Chemical oxygen demand (total and soluble) and total VFA concentrations of pH-reduced piggery influents and thermophilic reactor effluents at 2-d HRT

Thermophilic piggery wastewater	Total COD (mg/L)	Soluble COD (mg/L)	Total VFA (mg COD/L)
Influent (medium)	7341 (355)	5031 (153)	4906 (179)
Effluent (medium)	6956 (784)	4950 (431)	4427 (228)
Influent (high)	13220 (761)	7010 (910)	7687 (649)
Effluent (high)	12326 (855)	8423 (558)	8571 (447)

Data are mean values (± standard deviation)

While the COD (total and soluble) concentrations of the mid-strength influent and effluent were not statistically different ($p>0.05$), there was a small significant reduction (9 ± 4%) of influent total VFA concentration mainly from acetate, n-butyrate, i-valerate, n-valerate and caproate (Figure 6.19). No significant differences were observed between the influent and effluent propionate and i-butyrate concentrations (Table 6.22).

Table 6.22. Volatile fatty acid concentrations of the piggery influents and thermophilic reactor effluents at 2-d HRT

Thermophilic piggery wastewater	Acetate (mg COD/L)	Propionate (mg COD/L)	i-butyrate (mg COD/L)	n-butyrate (mg COD/L)	i-valerate (mg COD/L)	n-valerate (mg COD/L)	Caproate (mg COD/L)
Influent (mid)	2134 (61)	1019 (31)	198 (48)	572 (16)	494 (15)	375 (16)	114 (10)
Effluent (mid)	1828 (89)	1009 (64)	220 (50)	519 (28)	465 (21)	336 (35)	90 (5)
Influent (high)	3409 (255)	1830 (197)	311 (101)	674 (34)	764 (54)	508 (35)	190 (7)
Effluent (high)	3605 (7)	2159 (147)	488 (72)	717 (26)	857 (48)	523 (22)	222 (35)

Data are mean values (± standard deviation)

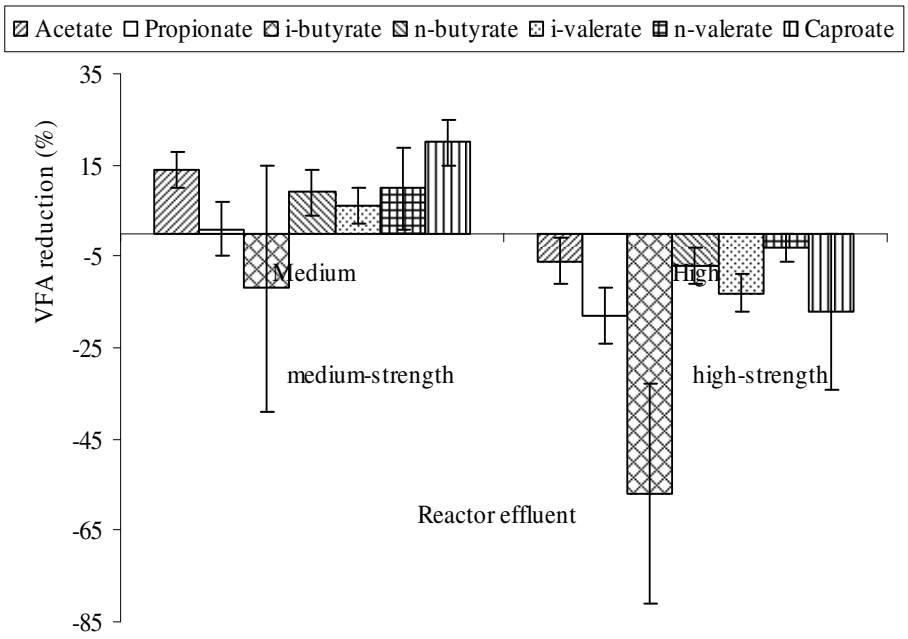


Figure 6.19. Percentage reduction or increase of volatile fatty acids in the thermophilic digested effluents relative to influent (error bars indicate standard deviations)

In contrast to the medium-strength piggery influent, the high-strength pH-adjusted piggery influent showed significant ($p<0.05$) increase in soluble COD and total VFA concentration following treatments in the anaerobic thermophilic reactor (Table 6.22). VFA analysis of the influent and effluent revealed that all the effluent C2 to C6 VFAs showed significant elevation over the influent as shown by their negative percentage reductions in Figure 6.19. The increased total VFA and soluble COD concentrations were consistent with the increased VSS reduction of the piggery influent which suggested that hydrolysis and acidification of the particulate organic matter had been enhanced.

Trace amount of methane (1%) was detected in the biogas from the anaerobic reactor treating medium-strength piggery influent (Table 6.23). However, no detectable methane except trace amount of hydrogen (1%) and carbon dioxide (44%) was detected in the biogas from the reactor treating high-strength wastewater. The balance was assumed to be nitrogen which could not be measured as high-purity nitrogen was used as the GC column carrier gas.

Table 6.23. Biogas composition and methane yields from the thermophilic first-stage anaerobic reactors treating medium- and high-strength piggery wastewaters with pH-reduction

Organic loading rate (gTCOD/L/d)	% methane in biogas	% hydrogen in biogas	% carbon dioxide in biogas	Methane yield (m3/kg TCOD fed) at 55^{o}C
3.71 (medium)	1 (1)	nd	44 (6)	0.001 (0.002)
6.61 (high)	nd	1 (0)	43 (2)	0

Data are mean values (± standard deviation) nd (not detected)

COD material balance

Figure 6.20 shows the COD material balance constructed from the COD components present in the medium- and high-strength pH-reduced influents and effluents relative to their respective influent TCOD concentrations.

The medium-strength piggery influent VFAs were observed to be largely conserved after anaerobic treatment in the first-phase reactor at 2-day HRT. The loss of about 10% influent VFA was largely accounted for as other unknown soluble organic matter in the digested effluent. Unlike the diluted medium-strength influent, further hydrolysis of the high-strength influent particulate organics and acidification of the soluble organics occurred during anaerobic treatment in the thermophilic reactor and all the VFAs were conserved.

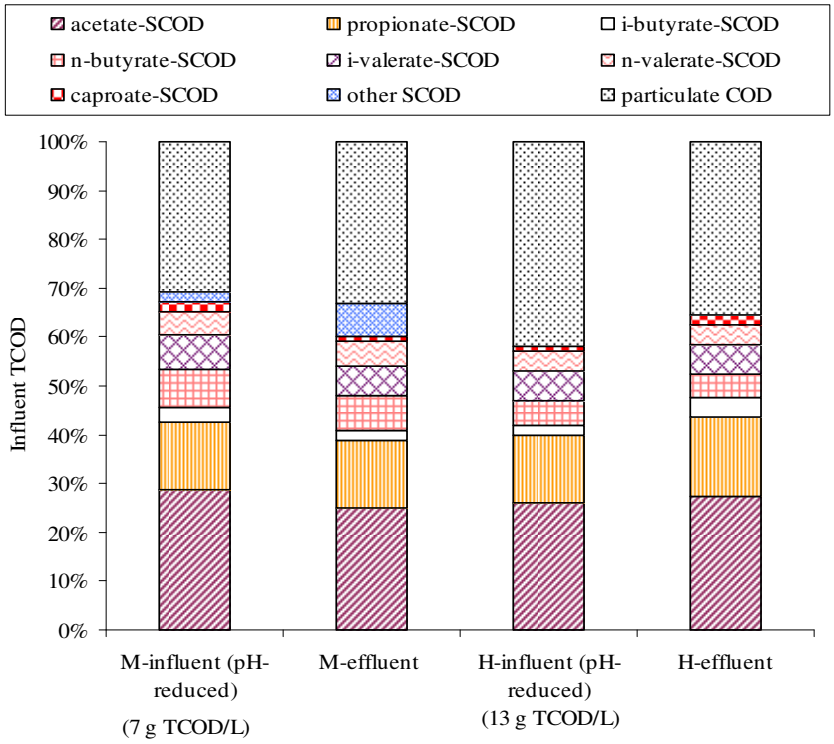


Figure 6.20. COD material balance of the medium- (M) and high-strength (H) piggery influents with pH reduction and thermophilic reactor effluents (medium- and high-strength)

Overall, the COD material balance of the medium- and high-strength thermophilic effluents show that around 35% of the organic matter remained as non-degradable particulate organics while 64-67% existed as soluble organics primarily in the form of VFAs in the medium-strength effluent and wholly VFAs in the high-strength effluent. Acetate was the predominant VFA, followed by propionate.

Reactor performance in the conversion of organics

Data on the organics conversion of the medium and high organic strength piggery wastewaters in Table 6.24 show that while there was negligible net hydrolysis of the medium-strength particulate organics taking place in the anaerobic thermophilic reactor, further hydrolysis of the high-strength particulate organics and conversion of the soluble organics to VFAs occurred.

Table 6.24. Comparison of the degree of hydrolysis, acidification, methanogenesis and hydrogenogenesis in the first-stage anaerobic reactors treating medium- and high-strength piggery wastewaters with pH reduction

Parameter	Unit	Effluent	Effluent
Piggery water strength		Medium	High
Degree of net hydrolysis	% influent TCOD	2 (3)	12 (9)
Degree of net acidification	% influent TCOD	0	7 (2)
Degree of methanogenesis	% influent TCOD	0.03 (0.04)	0
Degree of hydrogenogenesis	% influent TCOD	0	0.01 (0.01)

Data are mean values (± standard deviation)

Trace amount of methane was produced from the reactor treating the medium-strength influent. However, no detectable methane was observed in the biogas from the reactor treating the high-strength influent except trace amount of hydrogen.

Anaerobic microbial populations

Table 6.25 gives the FISH estimates of *bacteria* and *archaea* populations present in the medium- and high-strength pH-reduced piggery influents and reactor effluents. Despite the piggery wastewater being acidic at around pH 5.5, viable methanogenic *archaea* were still being detected in the digested thermophilic effluents in high numbers as shown in Figures 6.21 (a) and 6.22 (a).

Table 6.25. Quantification of bacteria and methanogen populations in the medium and high-strength pH-adjusted piggery influents and thermophilic digested effluents using 16S rRNA-specific FISH probes of EUBMIX and ARC-915 respectively

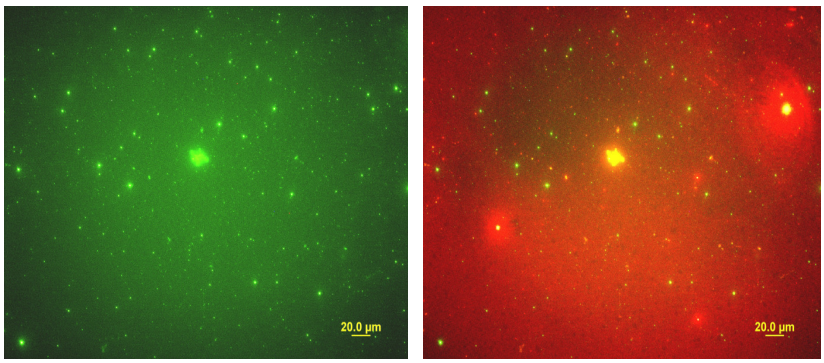
pH-reduced piggery wastewater	*Bacteria* (counts/ml)	*Archaea* (counts/ml)
Influent (medium)	$(4.61 \pm 0.98) \times 10^8$	$(5.90 \pm 0.96) \times 10^8$
Effluent (medium)	$(3.18 \pm 0.21) \times 10^8$	$(7.18 \pm 0.53) \times 10^8$
Influent (high)	$(1.56 \pm 0.20) \times 10^9$	$(5.52 \pm 0.72) \times 10^8$
Effluent (high)	$(1.56 \pm 0.09) \times 10^9$	$(6.02 \pm 1.39) \times 10^8$

Data are mean values of replicate measurements (± standard deviation)

The numbers of *bacteria* and methanogenic *archaea* in the high-strength piggery influent were observed to remain relatively constant after thermophilic reactor treatment at 2-d HRT, with the bacteria number significantly ($p<0.05$) greater than

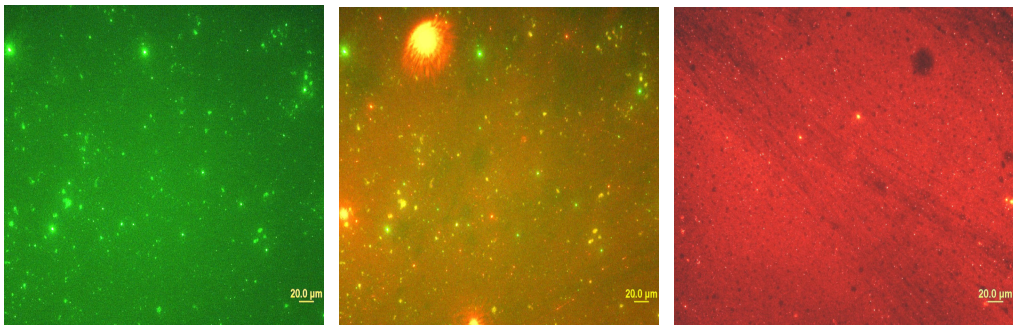
the *archaea* number. In the case of the medium-strength wastewater, while the influent had comparable levels of *bacteria* and *archaea*, the digested effluent showed an increase in *archaea* number and a corresponding decrease in bacteria number. The higher *archaea* number over the *bacteria* number seemed at odds with the fact that fermentative bacteria (hydrolytic and acidogenic) within the *bacteria* domain have the shortest doubling time. Nonetheless, the higher *archaea* level corresponded with the production of trace amount of methane (Table 6.23) while the lower bacteria number corresponded to the negligible hydrolysis of the particulate organics (Table 6.24).

(a) (b)



Figures 6.21 (a) and (b). Fluorescent images of *archaea* (green) and *archaea* (green) plus total *bacteria* (red) in thermophilic reactor effluent (pH-reduced medium-strength) respectively

(a) (b) (c)



Figures 6.22 (a), (b) and (c). Fluorescent images of *archaea* (green), *archaea* (green) plus low-GC bacteria (red) and total *bacteria* (red) in thermophilic reactor effluent (pH-reduced high-strength) respectively

T-RFLP profiles generated using *archaeal* primers identified the predominant methanogens in the mid- and high-strength influents and digested effluents to belong to the hydrogenotrophic *Methanoculleus spp.* (Table 6.26).

Table 6.26. T-RFLP molecular profile results of piggery influents with pH reduction and thermophilic digested effluents at 2-d HRT

Piggery wastewater	*Methanoculleus* spp. (%)	*Methanosarcina acetivorans* (%)	*Methanosarcina thermophila* (%)	Others
pH-reduced influent (medium)	90		10	
Effluent (medium)	95-99		5	1
pH-reduced influent (high)	60	25	5	
Effluent (high)	90-95	5-10		

To gain an insight into the phylogenetic microbial types within the *bacteria* domain, samples from the high-strength piggery influent and digested effluent were selected for further FISH study. Group-specific oligonucleotide FISH probes targeting alpha-, beta- and gamma-proteobacteria as well as the low- and high-GC bacteria were used to identify and quantify the different bacteria groups. The abundance of each specific group of bacteria was expressed as percentage of the total *bacteria* domain. Figure 6.23 illustrates the distribution of various phylogenetic groups of *bacteria* in the high-strength piggery wastewaters.

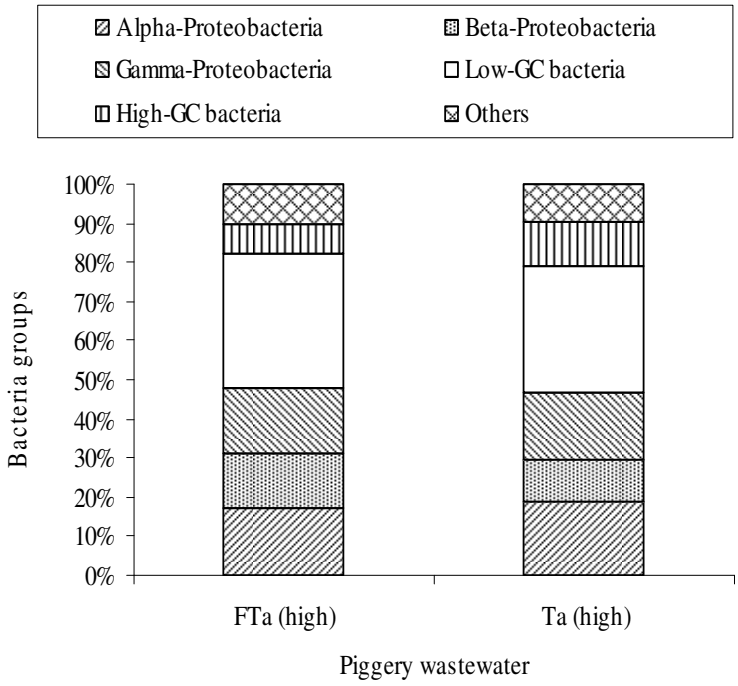
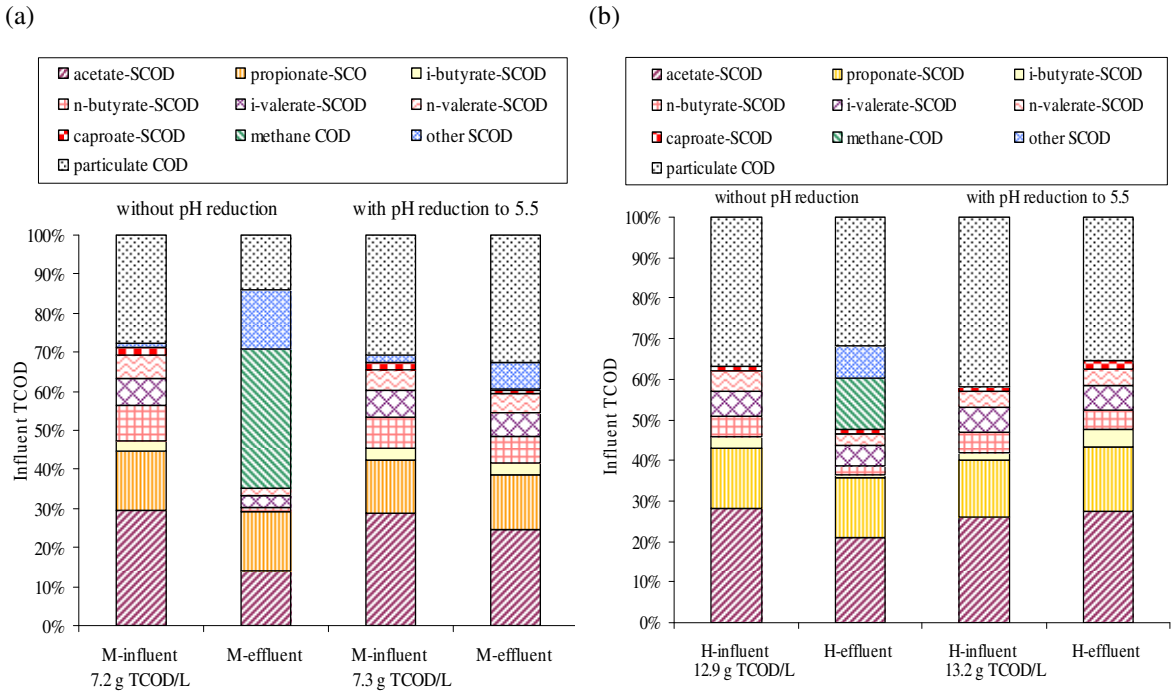


Figure 6.23. Distribution of various phylogenetic groups of *bacteria* in the high-strength piggery influent with pH reduction (FTa) and digested effluent (Ta)

Low-GC bacteria were the largest group (33%) in the *bacteria* domain of the pH-reduced piggery influent and effluent, followed by alpha-proteobacteria (19%), gamma-proteobacteria (17%), beta-proteobacteria (11%), high-GC bacteria (11%) and others (10%). The proportions of the different bacteria groups were similar between the influent and effluent. Appendices 1 and 2 list the bacteria genera that belong to the phylogenetic groups of Gram-negative proteobacteria and Gram-positive G+C bacteria.

6.3.2.3. Performance comparison of the thermophilic (55°C) first-stage anaerobic reactors treating medium- and high-strength piggery wastewaters with and without pH-reduction

Figures 6.24 (a) and (b) compare the COD material balance of the pH-unadjusted and pH-reduced piggery wastewaters of medium- and high-TCOD strength respectively while Figures 6.25 (a) and (b) compare the viable anaerobic microorganisms present in the medium-strength and high-strength piggery wastewaters with and without pH reduction respectively.



Figures 6.24 (a) and (b). COD material balance of mid-strength (M) and high-strength (H) piggery wastewaters respectively

Without pH reduction, some of the soluble organic substrates as VFAs (and hydrogen) were lost as methane and carbon dioxide during first-phase anaerobic treatment of the medium-and high-strength piggery wastewaters. More VFAs were lost when the wastewater was diluted to medium-strength which corresponded with a significantly higher methane production and *archaea* number (Figure 6.15 (b), Figures 6.26 (a) and (b)) than in the high-strength effluent due possibly to the reduction of the toxic free ammonia concentration (Table 6.10). While the medium-strength wastewater without pH reduction had significantly higher net extent of solubilisation than its pH-reduced counterpart (Figure 6.25) despite their bacteria numbers being comparable (Figure 6.26 (a)), there was no significant difference in the net extent of solubilisation between the high-strength wastewaters with and without pH reduction despite the bacteria number in the pH-reduced effluent being significantly higher (Figure 6.26 (b)). However, the net extent of acidification was significantly higher in the pH-reduced high-strength wastewater than its counterpart without pH reduction.

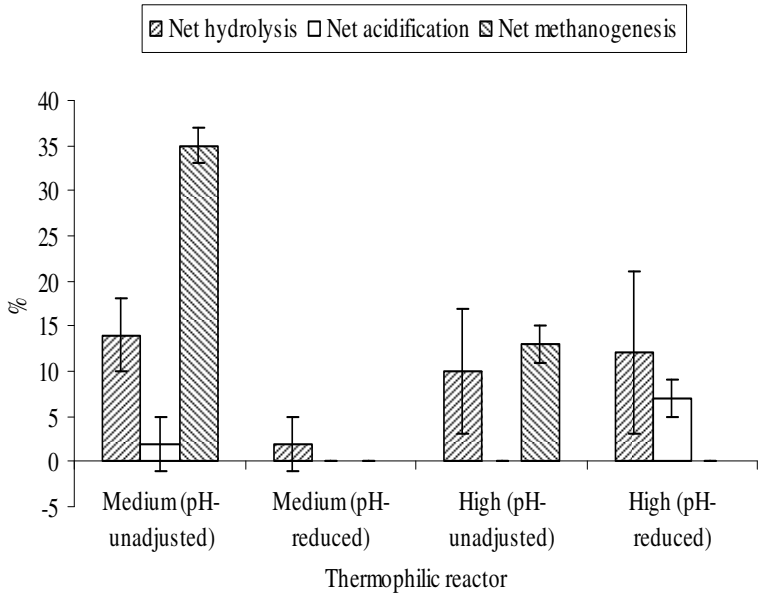
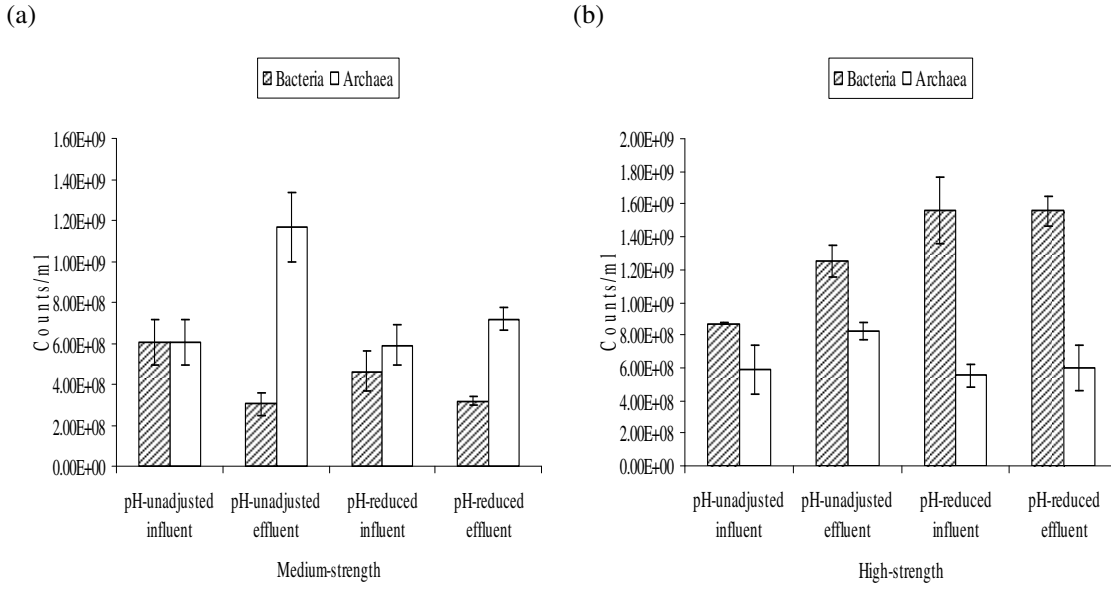


Figure 6.25. Comparison of the extent of net hydrolysis, acidification and methanogenesis of the thermophilic anaerobic reactors treating pH-unadjusted and pH-reduced piggery wastewaters (error bars indicate standard deviations)

Depending on the feed concentration or organic loading rate, pH reduction of the piggery wastewater resulted in almost entire to entire of the soluble organic substrates as VFAs being conserved while negligible to hardly any detectable

methane was produced. The results clearly demonstrated the effectiveness of pH reduction in inhibiting the syntrophic acetogenic and methanogenic microorganisms.



Figures 6.26. (a) and (b). Anaerobic *bacteria* and *archaea* populations in the mid-strength and high-strength piggery wastewaters respectively (error bars indicate standard deviations)

Surprisingly, the *archaea* numbers in the medium-strength piggery wastewaters with and without pH reduction after reactor treatment were noted to be substantially higher than the bacteria numbers (Figure 6.26 (a)). While in the high-strength piggery wastewaters with and without pH reduction, the bacteria numbers were substantially higher than the *archaea* numbers after reactor treatment (Figure 6.26 (b)). It is unclear whether rduction of the high-strength wastewater toxicity through dilution had played a part in improving the environmental conditions of the medium-strength wastewater for the *archaea*.

Reduction of the piggery wastewater pH to 5.5 appeared to increase the proportion of beta-proteobacteria over the piggery wastewater without pH reduction while the proportions of low-GC bacteria and unidentified other bacteria were reduced somewhat (Figure 6.27).

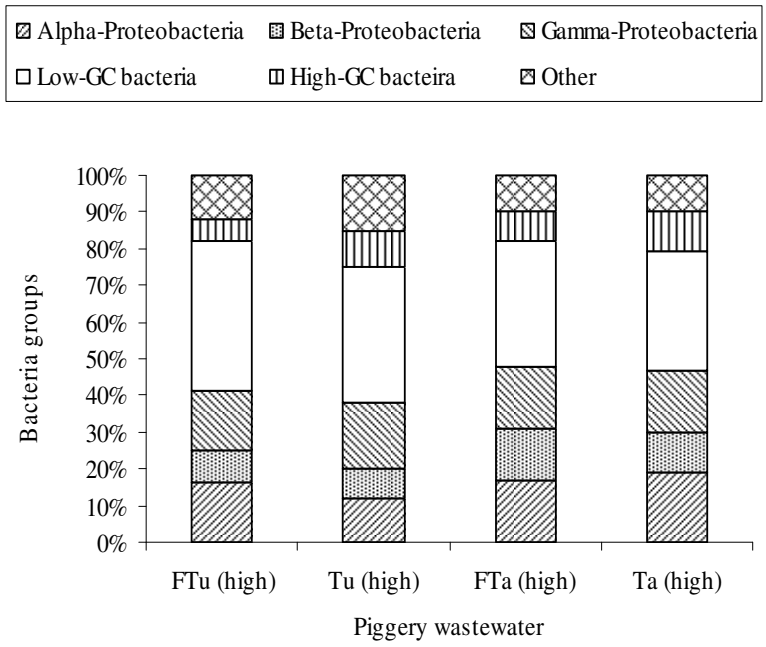


Figure 6.27. Comparison of the distribution of phylogenetic *bacteria* groups in the pH-unadjusted (FTu) and pH-reduced (FTa) high-strength piggery influents as well as their thermophilic effluents (Tu and Ta) respectively at 2-d HRT using 16S rRNA-group specific FISH probes

6.4. DISCUSSION

In the three sets of experimental results in sections 6.3.1.1, 6.3.2.1 and 6.3.2.2, inconsistencies between TCOD and SCOD or TVFA-SCOD reductions as well as between SCOD and total VFA-SCOD reductions were commonly observed. The cause was attributed largely to spatial variation in the distribution of particulate organic matter in the whole sample for TCOD determination and the presence of non-VFA components in the soluble organic fraction for SCOD determination. The calculated SCOD reduction data based on VFA analysis was considered most reliable of the three COD reduction data as the gas chromatography method used for VFA determination exclude the non-VFA soluble components.

Another common observation was the consistently lower actual specific methane yields compared to the theoretical specific methane yield in the first two sets of experimentals described in sections 6.2.1 and 6.2.2 (without pH adjustment). A number of factors could have contributed to the lower values. These include measurement errors in gas and VFA-COD determinations as well as possible microbial competition for methanogenic substrates (acetate, hydrogen plus carbon

dioxide). Anaerobic microorganisms that can compete with the methanogenic *archaea* include sulphate-reducing bacteria (SRB) and homoacetogens for VFA and hydrogen (section 2.2.5 in Chapter 2). In the case of SRB, the reduction of 1.5 g of SO_4^{2-} theoretically consumes 1.0 g of COD since 1 mole of SO_4^{2-} (96 g) requires two moles of oxygen (64 g) for its reduction to sulphide (de Lemos Chernicharo, 2007). This means less COD is available for conversion to methane. For the high-strength piggery wastewater, the limited sulphate data of the influent and effluent (Table 6.10) suggested that 0.61 g SO_4^{2-} had been reduced. This amount would theoretically consume 0.407 g COD which made it unavailable for the production of 0.142 L of methane based on the theoretical specific methane yield of 0.35 L per gram COD removed.

6.4.1. Performance comparisons of the thermophilic (55^{o}C) and mesophilic (37^{o}C) first-stage anaerobic reactors treating low-strength piggery wastewaters with synthetic complex wastewaters at 2-day hydraulic retention time

It was revealed during routine pH monitoring of the reactor effluents that the diluted thermophilic and mesophilic piggery wastewaters (4-5 g/L TCOD) were very resistant to pH changes due to their high buffering capacity as reflected by the high alkalinity level (Table 6.1). The high buffering capacity was provided by the bicarbonate and ammonium-nitrogen released during anaerobic degradation of the nitrogen-containing urea and proteinaceous materials in the piggery wastewater. These alkaline components served to neutralise the organic acids formed during anaerobic digestion (Thauer *et al*., 1977; McInerney, 1988; Gallert *et al*., 1998). In contrast, the rapid pH drop of the synthetic complex wastewater clearly demonstrated that the wastewater contained negligible alkalinity to buffer it against the acidity of the organic acids formed during fermentation.

Their vast differences in physico-chemical (Table 6.9) and microbiological (Figures 6.7 (a) and (b), Figures 6.8 (a) and (b)) characteristics explained the observed differences in fermentation products and biogas (Figures 6.9 (a) and (b)) produced in the anaerobic first-stage reactors under the same process conditions of 55^{o}C and 2-d HRT. They also explained why the thermophilic and mesophilic anaerobic reactors treating the piggery and synthetic wastewaters displayed vast differences in their organics conversion efficiency (Figures 6.10 (a) and (b)). The slightly alkaline pH

and the abundance of acidified soluble substrates (Table 6.9) in the diluted piggery wastewater provided a conducive environment for the growth of the syntrophic association of VFA-consuming acetogenic and methanogenic microbial populations. Their presence were strongly demonstrated by the significant reductions in acetate, propionate, n-butyrate and n-valerate concentrations (Figure 6.2) and the production of methane plus carbon dioxide (Table 6.5) in the thermophilic and mesophilic first-stage anaerobic reactors. The presence of methanogens was confirmed by T-RFLP molecular profiling (Table 6.8) which identified them to be predominantly *Methanosarcina acetivorans* and *Methanoculleus spp*. in the mesophilic reactor effluent and *Methanosarcina thermophila* in the thermophilic reactor effluent. The observed broader methanogenic diversity in the mesophilic reactor effluent concurred with the findings of other researchers (Leven *et al*., 2007; Karakashev *et al.*, 2005; Sekiguchi *et al.*, 1998).

In the case of the synthetic complex wastewater with low soluble organics and ammonia-nitrogen (Table 6.9), the rapid process souring to pH around 4 following microbial conversion of the soluble organic materials to volatile fatty acids showed the synthetic wastwater had minimal alkalinity to buffer it against VFA souring. The total lack of methane except small amount of hydrogen was clear evidence that the methanogens whose preferred pH range is between 6.7 and 7.2 (Novaes, 1986) and whose presence were positively confirmed by molecular FISH analysis (Figure 6.7 (b)) had been completely inactivated by the low pH environment. The observation of viable *archaeal* cells in the acidogenic anaerobic reactor was consistent with previous study which found archaeal population of 10^6 in the anaerobic acidogenic reactor that produced no methane at 0.5-day HRT (Yu *et al*., 2005). At low HRT operating conditions, hydrogenotrophic methanogens have a competitive advantage over the acetoclastic methanogens due to their higher growth rates (μ_{max} = 1.32 – 1.44 d^{-1}) or lower doubling times of between 4 to 11 hours (Yu *et al*., 2005; Zhang and Noike, 1991).

It is doubtful that had the synthetic wastewater pH being adjusted with sodium bicarbonate to provide it with some alkalinity instead of with sodium hydroxide, it would have altered the microbial composition and the resultant fermentation products to those closer to that of the real piggery wastewater. It is important to note

that one major contrast between the synthetic wastewater and the piggery wastewater was that the piggery wastewater contained a cocktail of high ammonia-containing urine and faeces which were the by-products of fermentation of the commercial food pellets excreted from the pig rumen. The latter itself functions as a natural anaerobic digester reactor with its own microflora. Furthermore, the piggery wastewater had also undergone further fermentation by the facultative and anaerobic micro-organisms found in the pig faeces in the on-site piggery holding sump before being treated in the first-stage anaerobic reactors.

In contrast, the synthetic organic wastewater was made up of commercial pig food pellets prepared in clean deionised wastewater which had not undergone any prior fermentation in any form. It also did not harbour any natural anaerobic microflora (Figures 6.7 (a) and (b)) and required initial seeding with municipal wastewater anaerobic sludge which contained indigeneous microorganisms. The observed differences in the phylogenetic distribution of the bacteria groups between the piggery wastewater (Figure 6.8 (a)) and the synthetic complex wastewater (Figure 6.8 (b)) were therefore not surprising.

Although species identification was not determined on the phylogenetic bacterial groups, the reduction of gamma-proteobacteria proportion which could contain the pathogenic *E. coli*, *Enterobacter*, *Salmonella* in the thermophilic piggery effluent was consistent with previous studies which found greater reduction in faecal coliforms, enterococcus and *Salmonella spp.* in sewage sludge at thermophilic temperature of 55^{o}C compared to mesophilic temperature of 35^{o}C (Watanabe *et al*., 1997).

Lower proportion of gamma-proteobacteria was also observed in the synthetic wastewater treated at thermophilic temperature of 55^{o}C compared to mesophilic temperature of 37^{o}C (Figure 6.8 (b)). In this case, protonated VFAs at low pH values which have been shown to kill bacteria and fungus in other studies (Salsali *et al*., 2008; Fukushi *et al*., 2003; Tenuta *et al*., 2002; Cherrington *et al.,* 1991; Lee *et al*., 1989) in conjunction with high temperature were likely factors responsible for the lower proportion of gamma-proteobacteria found in the synthetic wastewater. On the other hand, the increase in low-GC bacteria proportion in the thermophilic piggery and synthetic reactor effluents was more likely to be due to the increase number in

heat-resistant spore-forming, hydrogen-producing *Bacillus* and *Clostridium* genera which are known to be stimulated by heat. Previous studies by other researchers (Aiken *et al*., 2005b; Puchajda and Oleszkiewicz, 2003) have found that *Clostridium perfringens* spores were not removed to a significant extent at thermophilic conditions of 51-55^{o}C.

In summary, the results from this set of experiments demonstrated that unlike the synthetic organic wastewater, mesophilic and thermophilic first-stage anaerobic reactor treatment of the raw piggery wastewater at a short HRT of 2-day could not achieve complete acidogenic condition due primarily to its high natural buffering capacity. The findings from these sets of experiments highlighted the importance of using the right type of wastewater for laboratory experiments if the results were to be of practical relevance to the pilot-scale anaerobic acidogenic reactors treating piggery wastewater at SARDI.

6.4.2. Effects of piggery concentrations or organic loading loads (low, medium and high) without pH reduction on the performance of thermophilic (55^{o}C) first-stage reactors at 2-day hydraulic retention time

During the course of the reactor operations, it became increasingly apparent from the digested effluent pH that increasing the piggery influent concentration from 4 g/L TCOD or 2.2 g/L/d OLR to the maximum of about 13 g/L TCOD or 6.4 g/L/d OLR respectively had no effect in lowering the wastewater pH due to its high natural buffering capacity (Table 6.10). Increasing the piggery feedwater concentration or organic loading rate (OLR) merely released more ammonia-nitrogen (ammonium-nitrogen and free ammonia) and bicarbonate from urea and proteinaceous organics degradation. These products not only increased the pH of the raw wastewater from an initial pH of 7.3 to a final pH of 8.1, they also provided the piggery wastewater with an inherently strong buffering capacity against souring by VFAs formed during fermentation. Similar observation of high buffering capacity in the cattle manure wastewater was reported by Angelidaki and Ahring (1994).

The significant VFA reductions in the influent particularly acetate, n-butyrate, i- and n-valerate concentrations (Figure 6.12) and the concurrent production of methane plus carbon dioxide gas (Table 6.14) were evidence that the syntrophic fatty acid-

oxidising acetogenic bacteria and methane-forming methanogenic populations were active in the thermophilic anaerobic reactors treating the three different COD strength of the piggery wastewaters at 2-day HRT. Their presence was confirmed by both molecular FISH (Table 6.16) and real-time PCR analysis (Table 6.17).

Microbial counts by real-time PCR is widely known for yielding substantially higher results compared to the traditional cultivation-based methods such as dilution plating and most probable number (MPN) due to its inability to exclude the DNA from non-viable or dead cells (Lebuhn *et al*., 2005; Yu *et al*., 2005; section 2.5 in Chapter 2). While extensive comparative studies have been made between molecular real-time PCR and cultivation-based MPN methods (Lebuhn *et al*., 2005), it appears that there was a lack of comparative study being done between molecular real-time PCR and FISH on methanogens in particular, presumably due to the molecular FISH method is expected to yield considerably lower microbial counts than PCR method as the former only detects viable cells that contain rRNA.

The consistently higher *archaea* counts by FISH method over PCR method observed in Figure 6.14 (b) could be due to enhance visual sensitivity through the use of image processing software to detect *archaeal* cells with weak fluorescence intensity that would not have been detected otherwise on the visual images captured by the digital camera. Diaz *et al.* (2003) have reported difficulties in enumerating the different intensity positive hybridisation cells from non-hybridised cells which they comment could either overestimate or underestimate the cell counts. Karakashev *et al.* (2005) have also experienced difficulty with visual detection of some methanogens such as the hydrogenotrophic population and cited it as one of the limitations of molecular FISH method that could underestimate the actual *archaea* population. However, the consistently lower *archaea* counts by real-time PCR method compared to by FISH method in this study could be due to factors such as underestimation of the actual methanogen populations through the use of selected groups or species of methanogens to obtain the standard calibration graph and/or cell permeability issue with the molecular probes used (Skillman, personal communication).

T-RFLP profile analysis had identified the methanogenic populations in the low-, medium- and high-strength digested effluents to be predominantly hydrogenotrophic and acetoclastic *Methanosarcina thermophila* (Table 6.18). These methanogens

utilise hydrogen plus carbon dioxide, methanol, methyl amines and acetate as methaonogenic substrates to produce methane biogas and commonly dominate in anaerobic reactors operating at a short HRT and/or with high concentrations of acetate (>1 mM) and hydrogen (Leven *et al*., 2007; Leybo *et al.*, 2006; Yu *et al*., 2005; Diaz *et al*., 2003; Schmidt *et al.*, 2000; section 2.1.4 in Chapter 2). The acetate levels in the low-, medium- and high-strength piggery effluents were indeed all high: 12 mM (696 mg/L), 16 mM (941 mg/L) and 42 mM (2488 mg/L) respectively. Thus, the dominance of the *Methanosarcina thermophila* in the thermophilic digested piggery effluents was not surprisng.

However, the observed differences in the populations shift of the phylogenetic bacteria groups, in particular the Gram-positive low G+C group and the Gram-negative gamma-proteobacteria group in the diluted low-strength influent compared to the undiluted high-strength piggery influent was rather surprising (Figure 6.18). It is possible that dilution of the wastewater chemical components could have promoted the populations shift. The high proportion of Gram-positive low G+C bacteria group in the undiluted piggery influent was in agreement with the microbial findings of Snell-Castro *et al.* (2005) and Whitehead and Cotta (2001) on swine manure storage pit samples. Snell-Castro *et al.* (2005) reported that 74% of the bacteria phylogenetic group were from the low G+C group while Whitehead and Cotta (2001) found 79% to 100% of the 16S rDNA sequences, depending on pit depth were from the Gram-positive low G+C bacteria, with clostridial species being the predominant bacteria. Similarly, Iannotti *et al.* (1982) noted a predominately Gram-positive microflora in the swine waste anaerobic digesters. The higher species diversity observed in the bacterial group compared to the archeael group was in agreement with other researchers (Ariesyady *et al.*, 2007a; Leven *et al*., 2007; Mladenovska *et al*., 2003; Snell-Castro *et al*., 2005; Schmidt *et al*, 2000).

The reduction of the influent soluble phosphorus (Table 6.10) could be due to microbial uptake for cell synthesis while the reduction of sulphate was probably due to assimilation by sulphate-reducing bacteria (SRB). T-RFLP profiling had identified SRB of the genera *Desulfovibrio* to be present in the high-strength piggery effluent. This group can utilise lactate, pyruvate, acetate, carbon dioxide and certain fatty acids as carbon and energy substrates while reducing sulphate to hydrogen sulphide

(Stams *et al.*, 2003; Brock *et al.,* 1994). As SRB preferred pH environment lies between 7.5 and 8.0 (van Haandel *et al*., 2006), the high pH of the piggery wastewater therefore provided a favourable environment for their growth.

Microbial data from the molecular FISH (Table 6.16) and real-time PCR (Table 6.17) both indicate that dilution of the high-strength raw piggery wastewater to medium-strength had greatly stimulated the growth of methanogenic *archaea* over the *bacteria*. Incidientally, the high *archaea* number corresponded with the significantly ($p<0.05$) higher methane yield from thermophilic digestion of the medium-strength piggery wastewater over the high-strength wastewater (Figure 6.15). The results highlighted wastewater dilution as an effective strategry to enhance the methanogenic activity by reducing the high toxic free ammonia concentration in the raw piggery wastewater. However, in practice, this approach is considered undesirable on the basis of the increase in total volume of the piggery wastewater requiring anaerobic treatment.

Anaerobic digestion of animal wastes such as manure is indeed well known for producing toxic free ammonia and hydrogen sulphide gas which inhibit methanogenesis (Wiegant and Zeeman, 1986; Hansen *et al*., 1999; Angelidaki and Ahring, 1994). The severity of their inhibition depends on their concentrations, wastewater pH and microbial adaptation. Unionised free dissolved ammonia (NH_3-N) which is the toxic agent is produced in larger quantity than the ionised ammonium (NH_4^+) with increasing pH while unionised free hydrogen sulphide level increases with decreasing pH (Gerardi, 2006; Hansen *et al*., 1999; section 2.4 in Chapter 2). The significantly lower methane yield from the reactor treating pH-unadjusted high-strength piggery wastewater (OLR of 6.4 g TCOD/L/d) compared to the medium-strength wastewater (OLR of 3.5 g TCOD/L/d) concurred with Kim *et al.* (2004a) who reported that methane production rate and acidification drop when the OLR was above 5 g COD/L/d. It was likely that the methanogens had been inhibited by the higher ammonia concentration in the high-strength effluent (Table 6.10) as its total ammonia and free ammonia levels were above the threshold levels of 1.5-3.0 g-N/L and 700 mg-N/L respectively that cause inhibition of methanogenesis (McCarty, 1964; Angelidaki and Ahring, 1994).

In summary, the results from this set of experiments demonstrated that even at a relatively short HRT of 2-day, increasing the piggery wastewater organic strength to the maximum undiluted strength of 13 g/L TCOD could not prevent the syntrophic consortia of acetogens and methanogens in the wastewater from converting some of the methanogenic substrates to methane plus carbon dioxide. With the doubling times of acetogenic bacteria reportedly to be between 1.5 to 4 days, hydrogenotrophic methanogens between 4 to 11 hours and acetoclastic methanogens between 2 to 3 days respectively (section 2.2.4 of Chapter 2) coupled with the slightly alkaline effluent pH, it is clear that the first-stage anaerobic reactors operating at 2-day HRT without pH reduction was in effect functioning as conventional single-stage anaerobic reactors. In these reactors, fermentative hydrolytic and acid-forming acidogenic bacteria co-existed with the syntrophic acetogenic bacteria and methanogens as complex mixed culture rather than the intended acidogenic reactors which favoured the fermentative hydrolytic and acid-forming acidogenic bacteria.

6.4.3. Effects of pH reduction on organics conversion performance of thermophilic (55^{o}C) first-stage reactors treating diluted (medium-strength) and undiluted (high-strength) piggery wastewaters at 2-day hydraulic retention time

The elevated VFA concentrations (Table 6.22) in the undiluted high-strength digested effluent and a lack of methane in the reactor biogas (Table 6.23) clearly demonstrated that the pH reduction approach had stimulated the acid-producing acidogenic bacteria and successfully inhibited the syntrophic VFA-degrading acetogens and the predominantly hydrogenotrophic *Methanoculleus spp.* genus from consuming the methanogenic substrates (VFA, H_2 + CO_2) intended for the second-stage methane reactor. The unchanged soluble phosphorus in the reactor effluent (Table 6.19) suggested that other non-fermentative bacteria which utilised soluble phosphorus for cellular synthesis were also being inhibited by the acidic environment.

Inhibition of the microbial activities was attributed to increased toxicity of the acidic piggery wastewater due to changes to the chemical forms of potential toxicants such as hydrogen sulphide, VFA and long-chain fatty acid (LCFA) (Geraldi, 2006). At low pH, VFAs and hydrogen sulphide (H_2S) exist as unionised or protonated forms

which are more toxic than their ionised forms while LCFAs exist as the more toxic unsaturated form than the saturated form (section 2.5 in Chapter 2). Although H_2S and LCFA were not determined, they are commonly present in the animal wastewaters. For example, Snell-Castro *et al.* (2005) reported that the raw pig manure slurry (32 g/L SCOD) contained 326 mg/L H_2S. Sung and Santha (2001) found the biogas produced from thermophilic anaerobic digestion of dairy cattle (3 to 15 % TS) at 4-day HRT to contain 500 to 1300 mg/L H_2S. Hansen *et al.* (1998) found swine manure contained lipid content of 10.8 % (108,000 mg/L) and cattle manure contained 5.8% (58,000 mg/L) lipid.

For the diluted medium-strength piggery influent, the detection of trace level of methane in the biogas (Table 6.23) and reductions of acetate, n-butyrate, i- and n-valerate and caproate concentrations (Figure 6.19) in the reactor effluent were evidenced of a lesser degree of inhibition or toxicity on the syntrophic association of VFA-degrading acetogens and methanogens compared to the high-strength influent. The reduction of wastewater toxicity was a direct result of the dilution effect of wastewater that lowered the concentrations of inhibitory compounds such as the unionised VFAs and two potentially present toxicants of unionised H_2S and unsaturated LCFA which predominate at low pH (section 2.5 in Chapter 2). The trace amount of methane detected in the biogas (Table 6.23) and the increase in effluent *archaea* number (Table 6.25) which was identified by T-RFLP profiling to be predominantly hydrogenotrophic *Methanoculleus spp.* with very small proportion of acetoclastic *Methanosarcina* (Table 6.26) were further evidence of a reduced wastewater toxicity.

The results from this set of experiments demonstrated that a combination of high feed concentration or organic loading rate and pH-reduction to 5.5 could effectively suppress the activities of syntrophic VFA-degrading acetogens and methanogens and stimulate the hydrolytic-acidogenic fermentative bacteria. The inhibition of acetogens and methanogens by the acidic pH was likely to be due to a combined effect of the unionised hydrogen sulphide and VFA as well as the unsaturated LCFA as these components increase at pH value less than 6 (Geraldi, 2006; Komatsu *et al*., 1991; Hanaki *et al*, 1987). The unionised molecules of VFA and hydrogen sulphide diffuse more rapidly through the cell membrane of organisms than the ionised form

of VFA and sulfite (HS^-) respectively, lowering the internal pH of the microbial cells and disrupting their metabolic functions.

While pH reduction of the piggery wastewater effectively suppressed the methanogenic micro-organisms in the low HRT first-stage anaerobic reactor, it is important to note the practical problems associated with this approach. The dosing of concentrated hydrochloric acid to lower the highly-buffered wastewater pH to 5.5 will cause excessive foaming to occur as observed in the lab experiment which resulted in massive spill-over. As the concentrated acid is highly corrosive, great care needs to be taken to safeguard personnel health and safety during the acid handling on-site. Another drawback with this acid reduction approach is that the acidified reactor effluent will require pH readjustment back to around neutral with sodium hydroxide solution in order to safeguard the syntrophic acetogenic and methanogenic microorganisms from being severely inhibited in the second-stage methane reactor.

6.5. CONCLUSIONS

The results from the first experimental study showed that first-stage anaerobic treatment of low-strength piggery wastewater at 2-day HRT was unable to achieve complete acidogenic condition due to the combined effects of high alkalinity content in the piggery wastewater which neutralised the organic acids formed coupled with the operating HRT not low enough to flush out the syntrophic acetogenic and methanogenic microbial populations. Thus, the first-stage acidogenic anaerobic reactor had functioned as a conventional single-stage anaerobic reactor where hydrolytic and fermentative acidogenic bacteria co-existed with the syntrophic communities of VFA-consuming acetogenic bacteria, hydrogenotrophic and acetoclastic methanogens.

Due to their vast differences in the physico-chemical and microbiological characteristics, mesophilic and thermophilic anaerobic reactor treatment of the low-strength synthetic complex wastewater and the raw piggery wastewater at 2-day HRT yielded different fermentation products. While mesophilic first-stage anaerobic reactor treatment of the synthetic complex wastewater hydrolysed and acidified more organic matter to VFAs than the thermophilic first-stage anaerobic reactor, the reverse order was observed when raw piggery wastewater was treated in the

thermophilic and mesophilic anaerobic reactors. Thermophilic first-stage anaerobic reactor performed slightly better than the mesophilic reactor in terms of net hydrolysis, net acidification and methanogenesis of the organic carbon compounds in the piggery wastewater.

The results from the second experimental study highlighted that regardless of the feed concentrations or organic loading rates of the piggery wastewater, first-stage anaerobic treatment of the piggery wastewater without pH reduction could not prevent methanogeness from occurring at the short HRT of 2-day due to its high alkalinity which buffered the wastewater against VFA souring. Some losses of total VFAs still occurred at high TCOD feed concentration of 13 g/L or OLR of 6.5 g/L/d as the methanogenic microorganisms were not being completely inhibited. The lab-scale reactor observations provided useful insights into the conditions that existed in the pilot-scale anaerobic acidogenic reactors which operated at a longer HRT of 4 to 7-day during the pilot trial.

Results from the third experimental study revealed that while pH reduction of the undiluted piggery wastewater to pH 5.5 succeeded in completely inhibiting the syntrophic acetogenic-methanogenic microbial populations and stimulated the fermentative hydrolytic and acidogenic bacteria in the first-stage anaerobic reactor, the approach has its downsides to process operation. The excessive foaming which occurred during pH reduction of the piggery wastewater with concentrated hydrochloric acid resulted in messy spillages. In addition, the low effluent pH will certainly require pH readjustment back to neutral before it could be used as methanogenic substrate in the second-stage methane reactor. The resultant inconveniences are perceived to far out-weigh the small gains in the increased hydrolysis and acidification of the influent organic matter. Given that more than 60% of the organic carbon compounds in the highly buffered raw piggery wastewater had already been pre-fermented to volatile fatty acids at ambient conditions and that about 25% of the influent organic matter remained as undissolved particulate organics in the treated effluent coupled with its high buffering capacity against process souring, it was decided that the use of single-stage thermophilic anaerobic reactor with a sufficiently long HRT might be a more cost-effective option than a two-stage thermophilic-mesophilic anaerobic reactors for the treatment of the raw

piggery wastewater in terms of increasing the hydrolysis of the particulate organics including conversion of the bulk of the VFAs to methane plus carbon dioxide and pathogens control with a longer HRT.

CHAPTER 7
SEMI-CONTINUOUS SINGLE-STAGE ANAEROBIC DIGESTION OF RAW PIGGERY WASTEWATER AT HIGH HYDRAULIC RETENTION TIMES

7.1. INTRODUCTION

Previous first-phase semi-continuous anaerobic reactor experiments in Chapter 6 demonstrated that the highly-buffered piggery wastewater was very resistant to pH changes despite the reactors being run at a much shorter HRT of 2-day as opposed to the longer HRTs of 4- to 7-day of the pilot-scale reactors. Irrespective of the organic concentrations or organic loading rates tested, the laboratory-scale first-stage acidogenic reactors were in effect functioning as conventional single-stage anaerobic reactors, transforming part of the acetate and hydrogen plus carbon dioxide to biogas as methane plus carbon dioxide. These laboratory observations provided valuable insights into the conditions that existed in the pilot-scale acidogenic reactors with regards to the low VFA levels at 4- and 7-day HRT. Since the raw piggery wastewater had more than half of its organic compounds being acidified to VFAs at ambient conditions coupled with the fact that the first-stage digested effluent still had between 30 and 50% of its organics in particulate form, it was decided to conduct single-stage comparative studies on the digestion performance of thermophilic (55^{o}C) and mesophilic (37^{o}C) CSTR single-stage anaerobic treatment of the undiluted piggery wastewater at longer HRTs of 10- and 15-days.

The objectives of the semi-continuous anaerobic single-stage reactor experiments were to:

1) determine and compare the extent of hydrolysis of the particulate organics and conversion of volatile fatty acids to methane and carbon dioxide at 10-day and 15-day HRTs without pH adjustment under thermophilic conditions; and

2) compare the digestion performance of the thermophilic and mesophilic single-stage anaerobic reactors at 15-day HRT.

This chapter presents the experimental designs and findings from these laboratory-scale semi-continuous reactor experiments.

7.2. MATERIALS AND METHODS

7.2.1. Thermophilic (55^{o}C) single-stage anaerobic digestion of raw piggery wastewater at 10-day and 15-day HRT

Two 1.2-litres anaerobic reactors with working volumes of 0.9-litres were filled with digested piggery effluent from the previous thermophilic first-stage anaerobic reactor experiment on the pH-unadjusted, undiluted piggery effluent as described in section 6.2.2 of Chapter 6. The reactors were operated semi-continuously on a daily drain and feed mode at 55^{o}C to give 10-day HRT in one reactor and 15-day HRT in the other reactor. The general practice of assuming steady state after a minimum of three HRTs elapsed time was adopted. The reactors were fed with chilled undiluted piggery wastewater for a period of three to four volumes of hydraulic retention time before assumed steady state reactors' biogas and effluent were sampled for eight consecutive days. The same protocols of reactor sampling, analysis and data processing as the earlier experiments described in section 6.2.1.2 of Chapter 6 were adopted.

7.2.2. Mesophilic (37^{o}C) single-stage anaerobic digestion of high-strength piggery wastewater at 15-day HRT

Following completion of the thermophilic reactor experiment at 15-day HRT, the reactor content was emptied out and cleaned prior to filling it with the digested effluent from the earlier mesophilic reactor experiment on the diluted piggery effluent as described in section 6.2.1 of Chapter 6. The mesophilic reactor was operated on a semi-continuous mode of daily draining and feeding with chilled undiluted piggery wastewater at 15-day HRT and 37^{o}C for a period of three volumes hydraulic retention time before assumed steady state biogas and effluent were sampled from the reactor for eight consecutive days. The same protocols of reactor operation, sampling, analysis and data-processing as the earlier thermophilic experiments described in section 6.2.1.2 were adopted.

7.3. RESULTS

7.3.1. Thermophilic (55°C) anaerobic digestion of high-strength piggery wastewater at 2-day, 10-day and 15-day HRT

For comparison purposes, data from the previous first-stage thermophilic reactor experiment on the undiluted high-strength piggery wastewater at 2-day HRT are included in all the Tables and Figures below alongside the data of 10- and 15-day HRT. However, data from the thermophilic pilot-scale reactors at 4- and 7-day HRTs are not included here because they are observed to be vastly inconsistent with the data trend of the lab-scale reactors, particularly with the total VFA, ammonium-nitrogen and methane concentrations. The inconsistencies of the pilot-scale data were attributed to a combination of the reported wide fluctuations in the characteristics of the different batches of piggery wastewater fed to the pilot-scale first-stage reactors and other operational variables. Appendices 3.1, 3.2 and 4.1, 4.2 give tabulations of the data of the pilot-scale piggery feedwaters and the first-stage reactor effluents at 7- and 4-day HRT respectively together with data of the lab-scale piggery feedwaters and reactor effluents at 2-, 10- and 15-day HRT to highlight their inconsistencies which preclude meaningful comparisons.

pH, alkalinity, ammonium-nitrogen and free ammonia

Table 7.1 gives the pH, total alkalinity, ionised ammonium-nitrogen and unionised dissolved free ammonia concentrations in the piggery influents and effluents of the thermophilic single-stage reactors operating at 2-day, 10-day and 15-day hydraulic retention times.

Table 7.1. pH, total alkalinity, ammonium-nitrogen and free ammonia concentrations of the piggery influents and thermophilic (T) effluents at 2-d, 10-d and 15-d HRT

Undiluted piggery wastewater	HRT (d)	pH	Total alkalinity (mg CaCO3/L)	NH_4^+-N (mg/L)	Free NH_3-N (mg/L)
Influent to T-reactor	2	7.3 (0.1)	5595 (488)	1800 (0)	4 (1)
T-effluent	2	8.1 (0.1)	6500 (165)	2150 (191)	788 (85)
Influent to T-reactor	10	7.7 (0.1)	4938 (159)	1941 (66)	10 (2)
T-effluent	10	8.3 (0.1)	5533 (142)	2111 (56)	920 (127)
Influent to T-reactor	15	7.7 (0.1)	4550 (0)	1977 (21)	10 (1)
T-effluent	15	8.4 (0.1)	5894 (8)	2083 (42)	1026 (74)

Data are mean values (± standard deviation)

Variations in the initial pH and alkalinity were observed in the piggery influents used for the HRT experiments. These were attributed to the different start time of the reactor experiments which contributed to some changes in the characteristics of the piggery wastewater despite it being stored in the fridge during the duration of this project. Free ammonia concentrations at the longer HRTs of 10- and 15-day were generally higher than the short HRT of 2-day due to the higher effluent pH.

Total and volatile suspended solids (TSS and VSS)

Table 7.2 gives the total solids (TS), volatile solids (TS), total suspended solids (TSS) and volatile suspended solids (VSS) data before and after anaerobic treatment while Figure 7.1 illustrates the amount of solids removed.

Table 7.2. Solid concentrations in the undiluted influents and thermophilic (T) digested effluents at 2-d, 10-d and 15-d HRT

Undiluted piggery wastewater	HRT (d)	TS (g/L)	VS (g/L)	TSS (g/L)	VSS (g/L)
Influent to T-reactor	2	na	na	3.5 (0.8)	3.2 (0.7)
T-effluent	2	na	na	2.7 (0.5)	2.3 (0.6)
Influent to T-reactor	10	8.0 (0.1)	5.1 (0.1)	3.4 (0.2)	3.4 (0.5)
T-effluent	10	7.2 (0.2)	4.1 (0.2)	2.6 (0.2)	1.9 (0.2)
Influent to T-reactor	15	8.1 (0.2)	5.0 (0.2)	3.4 (0.1)	3.0 (0.1)
T-effluent	15	7.5 (0.2)	4.3 (0.2)	2.7 (0.2)	1.9 (0.2)

Data are mean values (± standard deviation) na (not available)

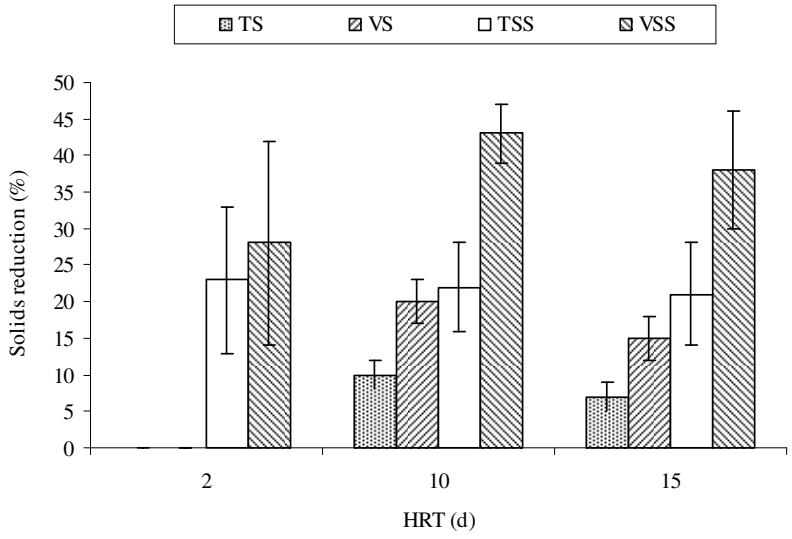


Figure 7.1. Solids reductions of thermophilic digested effluents at 2-d, 10-d and 15-d HRT (error bars indicate standard deviations)

At the longer HRT of 10 and 15-day, the amount of TS and VS removed were significantly ($p<0.05$) higher at 10-day than at 15-day HRT. While TSS removal was not significantly different ($p>0.05$) between 10- and 15-day HRT which indicated that hydrolysis of their particulate organics were comparable, VSS removal were significantly ($p<0.05$) higher at 10-day than at 15-day HRT.

COD, volatile fatty acids and biogas

Table 7.3 gives the COD (total and soluble) and total VFA-COD data of the piggery influents and thermophilic reactor effluents. Figure 7.2 illustrates the amount of COD removed from the thermophilic effluents after anaerobic treatment.

Table 7.3. Chemical oxygen demand (total and soluble) and total VFA concentrations of the undiluted piggery influents and thermophilic (T) digested effluents at 2-d, 10-d and 15-d HRT

Undiluted piggery wastewater	HRT (d)	Total COD (mg/L)	Soluble COD (mg/L)	Total VFA (mg COD/L)
Influent to T-reactor	2	12865 (774)	7619 (747)	8139 (340)
T-effluent	2	10365 (766)	7122 (422)	6009 (314)
Influent to T-reactor	10	10593 (464)	6129 (217)	5801 (115)
T-effluent	10	7924 (607)	4889 (196)	3452 (113)
Influent to T-reactor	15	11001 (562)	7270 (355)	5600 (304)
T-effluent	15	7151 (509)	4937 (405)	3026 (265)

Data are mean values (± standard deviation)

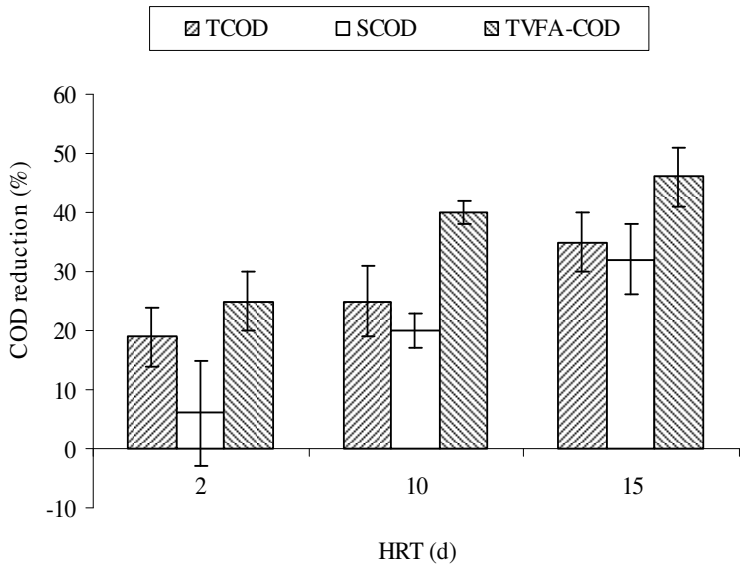


Figure 7.2. COD (total and soluble) and total VFA reductions of the thermophilic digested effluents at 2-d, 10-d and 15-d HRT (error bars indicate standard deviations)

It was observed that the initial TVFA concentration of the piggery influent used in the 2-day HRT reactor experiment was about 28% higher than in the piggery influents used in the 10-day or 15-day HRT. As mentioned earlier, the differences were attributed to the different start time of the experiments which resulted in some of the VFA components being consumed by the mixed anaerobic microorganisms in between the different HRT experiments (Table 7.4). With the variability of the wastewater being greater than the variables that arose from storage degradation, data comparisons were considered more meaningful using the percentage reduction data rather than the concentration data. Figure 7.2 shows that reductions in COD particularly soluble COD and TVFA-COD were as expected, lowest at 2-day HRT but highest at 15-day HRT which contrasted with the solid removal data illustrated in Figure 7.1.

Table 7.4 gives the volatile fatty acid components present in the digested effluents at 2-, 10- and 15-day HRT. The amount of VFA removal is illustrated in Figure 7.3.

Table 7.4. Volatile fatty acid concentrations of the undiluted piggery influents and thermophilic (T) digested effluents at 2-d, 10-d and 15-d HRT

Undiluted piggery wastewater	HRT (d)	Acetate (mg COD/L)	Propionate (mg COD/L)	i-butyrate (mg COD/L)	n-butyrate (mg COD/L)	i-valerate (mg COD/L)	n-valerate (mg COD/L)	Caproate (mg COD/L)
Influent to T-reactor	2	3619 (191)	1916 (111)	325 (45)	701 (19)	801 (31)	586 (77)	192 (4)
T-effluent	2	2652 (141)	1964 (169)	81 (91)	223 (19)	687 (25)	327 (19)	75 (88)
Influent to T-reactor	10	2042 (32)	1312 (55)	436 (5)	482 (6)	712 (13)	629 (58)	187 (28)
T-effluent	10	839 (82)	2067 (89)	152 (15)	24 (15)	208 (24)	66 (6)	96 (50)
Influent to T-reactor	15	2054 (87)	1334 (56)	451 (35)	498 (40)	698 (59)	431 (22)	134 (6)
T-effluent	15	631 (191)	2060 (79)	65 (18)	19 (19)	139 (41)	64 (21)	48 (3)

Data are mean values (± standard deviation)

Reductions of the influent acetate, n-butyrate, i- and n-valerate concentrations were higher at 10- and 15-day HRT than at 2-day HRT. However, propionate concentration was elevated by about two-fold at 10- and 15-day HRTs compared to at 2-day HRT.

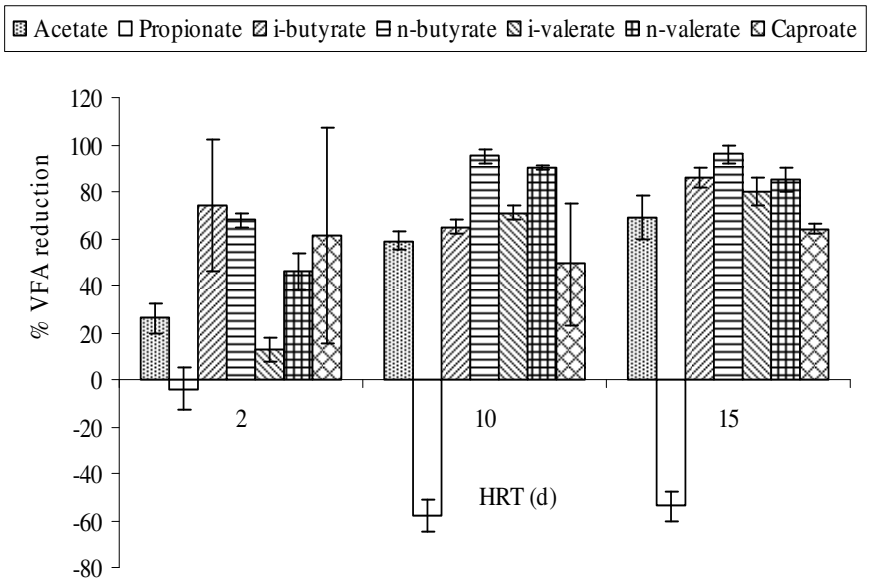


Figure 7.3. Percentage removal of VFA-COD in the digested effluents at 2-d, 10-d and 15-d HRT (error bars indicate standard deviations)

As ammonia is universally known for its microbial toxicity, the effect of free ammonia concentration on acetoclastic methanogens and propionate-degrading acetogenic bacteria was assessed at the three HRTs by plotting the VFA reduction data against their corresponding free ammonia concentrations. Figure 7.4 illustrates the relationships between free ammonia and the amount of acetate reduced as well as the amount of propionate accumulated.

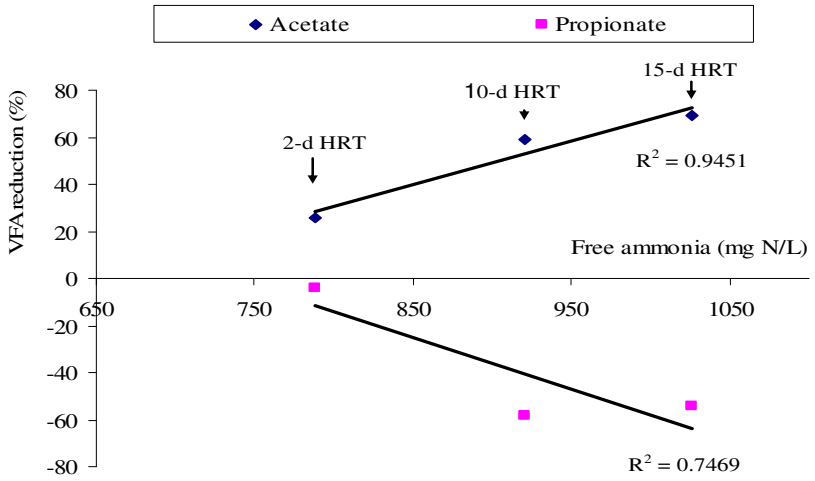


Figure 7.4. Relationships between acetate reduction, propionate increase and free ammonia concentration as a function of hydraulic retention time

A strong positive correlation (R^2 = 0.9451) was observed between free ammonia concentrations and the amount of acetate degraded. This indicated that the acetoclastic methanogens were not inhibited despite the effluent's high free ammonia with increasing HRT. In contrast, a negative correlation (R^2 = -0.7469) was observed between free ammonia concentrations and the amount of propionate accumulated with increasing HRT. The trend linked increasing free ammonia concentration to increasing amount of propionate accumulated, thus indicating that the syntrophic propionate-degrading acetogenic bacteria and hydrogenotrophic methanogens or SRB were more sensitive to the increased free ammonia concentrations with increasing HRT than the acetoclastic methanogens. Degradations of butyrate (i- and n-), valerate (i- and n-) and caproate were however, unaffected by the wastewater ammonia level as shown in Figure 7.3. The observation suggested that the butyrate-degrading acetogenic bacteria which oxidise butyrate and higher carbon fatty acids were more resilient to product inhibition than the propionate-degrading acetogenic bacteria.

Table 7.5 shows the biogas produced at 2-, 10- and 15-day HRT consisted of 71 to 73% methane and 21 to 30% carbon dioxide.

Table 7.5. Biogas composition and methane yield from the thermophilic reactors at 2-day, 10-day and 15-day HRTs

HRT (d)	Organic loading rate (g TCOD/L/d)	Total VFA-COD removal rate (g COD/L/d)	% methane	% carbon dioxide	Actual specific methane yield at stp (L/g COD removed)
2	6.433	1.024 (0.224)	72 (1)	30 (2)	0.229 (0.037)
10	1.059	0.235 (0.011)	73 (1)	21 (1)	0.286 (0.013)
15	0.733	0.172 (0.018)	71 (2)	24 (1)	0.322 (0.034)

Data are mean values of eight measurements (± standard deviation)

Statistical t-test analysis ($p<0.05$) of the methane yields showed 15-day HRT produced the highest methane yield, followed by 10-day and lastly 2-day HRT. The measured specific methane yields were generally lower than the theoretical values of 0.35 L/g COD removed. Possible reasons for the lower experimental values were as mentioned in section 6.4 of Chapter 6.

COD material balance

Figure 7.5 shows the COD material balance constructed from the COD components present in the undiluted piggery influents and thermophilic reactor effluents at 2-d, 10-d and 15-d HRT relative to their respective influent TCOD concentrations.

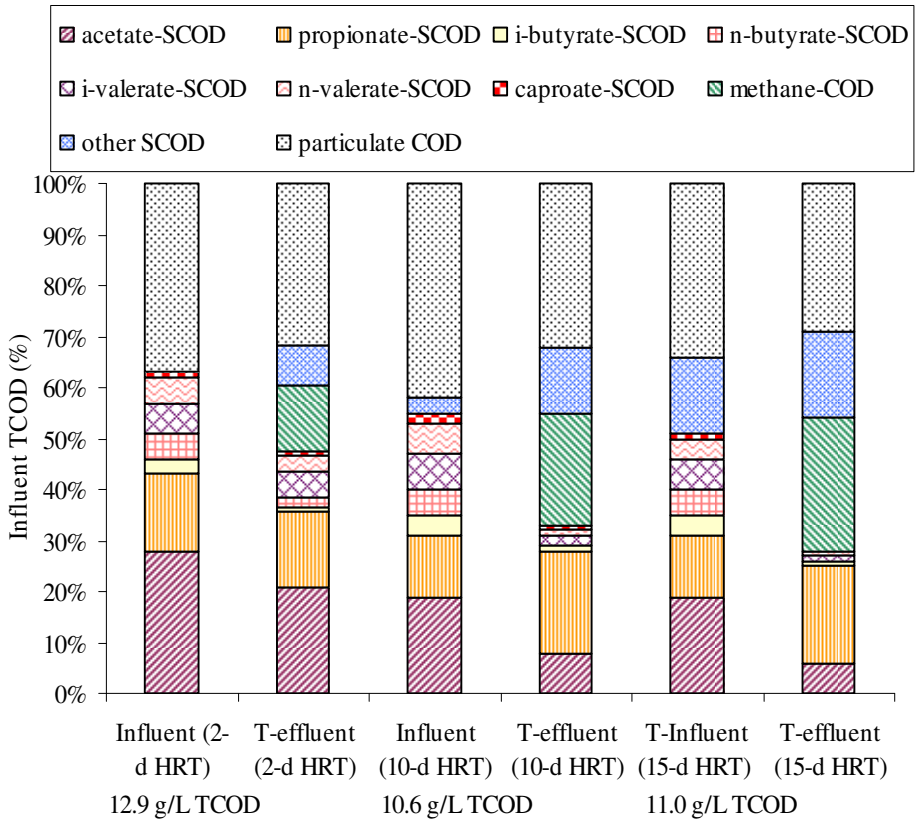


Figure 7.5. COD material balance of the undiluted piggery influents and thermophilic (T) reactor effluents at 2-, 10- and 15-day HRT

One distinct irregularity highlighted by the material balance was the large proportion of unidentified SCOD fraction (other SCOD) in the influent fed to the reactor at 15-day HRT. It was substantially more than its counterparts in the influents fed to the reactors operating at 2- and 10-day HRTs as well as in the influent fed to the mesophilic reactor at 15-day HRT (Figure 7.14). It is suspected that some fine particulate solids might have been dislodged during decanting of the centrifuged sample for SCOD measurement which resulted in an inflated result. As the VFA-COD components were separate measurements, they were not affected by the dubious influent SCOD data.

The COD material balance of the wastewaters shows that increasing the HRT from 2- to 10- and 15-day resulted in greater removal of VFAs except propionate and more methane being produced. Propionate was observed to accumulate at the longer HRT of 10- and 15-day than at the short HRT of 2-day. This indicated that the propionate-degrading acetogenic bacteria were under increased environmental stress. The proportion of other SCOD fraction was also observed to increase with increased HRT. The exception was 15-day HRT for reason given earlier.

Overall, the COD material balance of the thermophilic digested effluents show that around 30% of the organic matter remained as non-biodegradable particulate organics after anaerobic treatments at longer HRTs of 10- to 15-day. Propionate (19%) and unidentified non-VFA (13-17%) soluble organic matter formed the two largest groups of undigested soluble organics in the digested piggery effluents.

Reactor performance in the conversion of organics

Table 7.6 compares the digestion performance of the thermophilic anaerobic reactors at 2-, 10- and 15-day HRT. The data show that while additional hydrolysis of the influent particulate organic matter occurred in the thermophilic reactors, no significant ($p>0.05$) difference in the net hydrolysis was observed between 2-day and 10-day HRT. However, significant ($p<0.05$) difference was observed between 10-day and 15-day HRT, with the latter hydrolysed less of the influent particulate organic matter.

Table 7.6. Digestion performance data of the thermophilic reactors at 2-day, 10-day and 15-day HRT

HRT (days)	2	10	15
Extent of net hydrolysis (% feed TCOD)	10 (6)	10 (2)	5 (3)
Extent of net acidification (% feed TCOD)	0	0	2 (2)
Extent of methanogenesis (% feed TCOD)	13 (2)	22 (1)	26 (1)

Data are mean values (± standard deviation)

Although there was hardly any additional acidification of the soluble organic matter occurring at the three HRTs, increasing methane production was observed with increasing HRT. Highest extent of methane production was at 15-day HRT with 2-day HRT produced the least methane as expected.

Anaerobic microbial populations

Table 7.7 gives the bacteria and methanogen populations enumerated by molecular FISH method in the undiluted piggery influents and thermophilic digested effluents.

Table 7.7. Quantification of thermophilic (T) anaerobic microorganisms by domain oligonucleotide FISH probes of ARC-915 for *archaea* and EUBMIX for *bacteria*

Undiluted piggery wastewater	HRT (d)	Bacteria (cells/ml)	*Archaea* (cells/ml)
Influent to T-reactor	2	$(8.70 \pm 0.07) \times 10^8$	$(5.90 \pm 1.49) \times 10^8$
T-effluent	2	$(1.25 \pm 0.10) \times 10^9$	$(8.19 \pm 0.54) \times 10^8$
Influent to T-reactor	10	$(6.26 \pm 1.22) \times 10^8$	$(8.81 \pm 1.20) \times 10^8$
T-effluent	10	$(1.85 \pm 0.22) \times 10^9$	$(1.93 \pm 0.18) \times 10^9$
Influent to T-reactor	15	$(8.42 \pm 1.47) \times 10^8$	$(1.51 \pm 0.45) \times 10^9$
T-effluent	15	$(1.57 \pm 0.21) \times 10^9$	$(2.46 \pm 0.23) \times 10^9$

Data are mean values of replicates (± standard deviation)

It is noted that the piggery influents to the 10- and 15-day HRT thermophilic reactors had higher *archaea* numbers than the influent to the 2-day HRT thermophilic reactor whereas their corresponding bacteria numbers show no significant increase. The later start time of the reactor experiments at 10- and 15-day HRT probably had contributed to the higher *archaea* level in the piggery wastewater as some biogas was generated from the wastewater during storage in the fridge at 4^oC.

Nevertheless, both the increased bacteria and *archaea* numbers in the 10- and 15-day HRT thermophilic effluents were significantly higher than in the 2-day HRT effluent, with 15-day HRT effluent having the highest increased in *archaea* number. A plot of *archaea* number and extent of methanogenesis as a function of HRT (Figure 7.7) shows the highest methane yield at 15-day HRT corresponded with the highest *archaea* population in the 15-day HRT effluent.

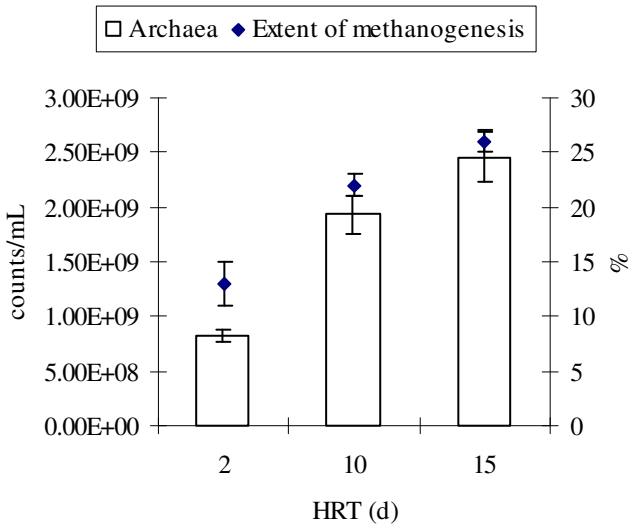
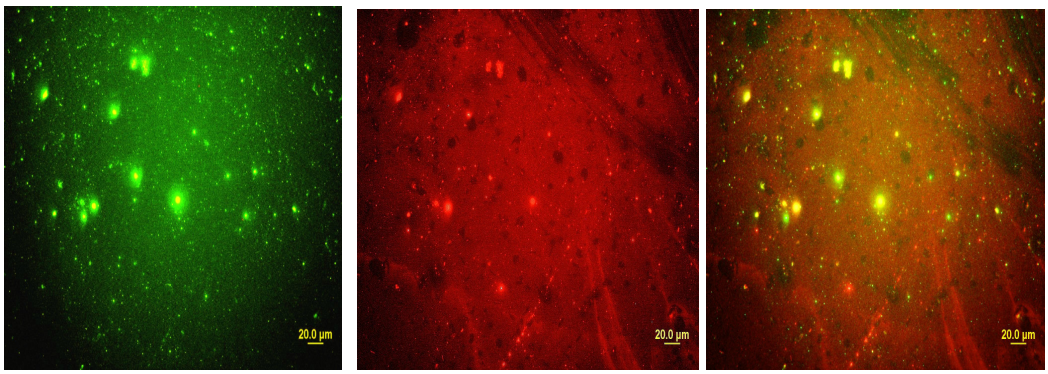


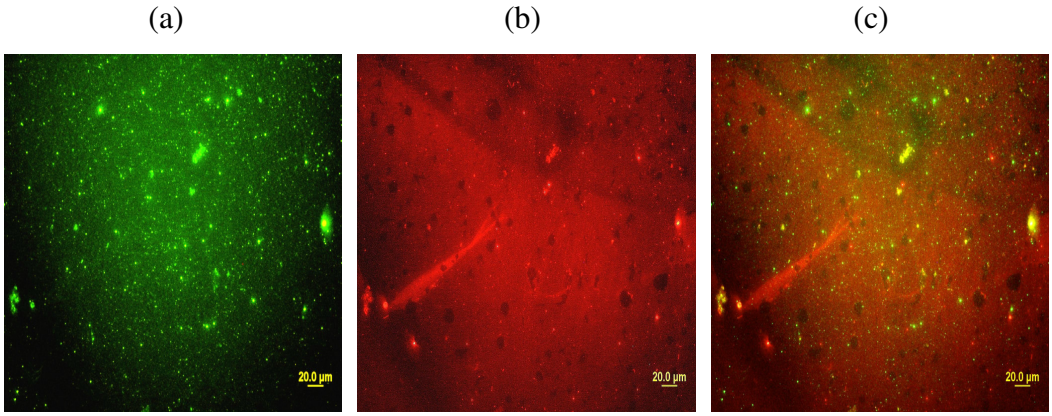
Figure 7.6. Relationship between *archaea* populations in the thermophilic reactor effluents and methane yields as a function of hydraulic retention time (error bars indicate standard deviations)

Figures 7.7 and 7.8 (a), (b) and (c) show the fluorescent images of the *archaea*, total *bacteria* (hydrolytic, acidogenic and acetogenic bacteria) and *archaea* plus *bacteria* in the digested effluents at 10- and 15-day HRT respectively.

(a) (b) (c)



Figures 7.7 (a), (b) and (c). Fluorescent images of themophilic *archaea* (green), bacteria (red) and superimposed image of (a) and (b) at 10-d HRT



Figures 7.8 (a), (b) and (c). Fluorescent images of thermophilic *archaea* (green), bacteria (red) and superimposed image of (a) and (b) at 15-d HRT

T-RFLP profiling of the bacterial communities in the piggery influent and thermophilic digested effluents (Figure 7.9) revealed greater microbial diversities in the digested effluents compared to the piggery feedwater. Appendix 5 lists the genera that belong to each bacterial group.

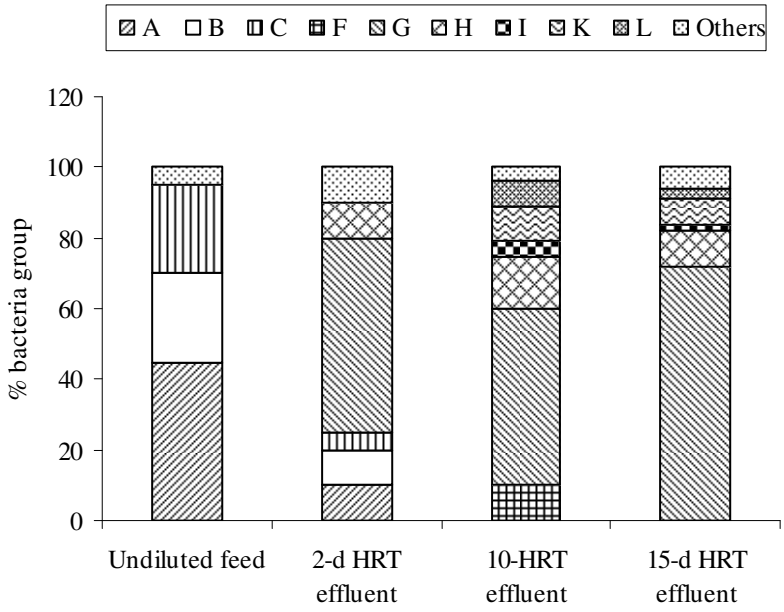


Figure 7.9. T-RFLP distribution profiles of the bacteria group in the undiluted piggery feedwater and thermophilic digested effluents at 2-, 10- and 15-day HRT

A gradual decline of Groups A, B and C microorganisms was observed in the digested piggery effluents after anaerobic treatment at 2-, 10- and 15-day HRT compared to the influent. While groups G and H were present at the three HRTs, additional groups of I, K and L were observed only at the longer HRT of 10- and 15-

day. Group G which includes the *Meiothermus Silvanus, Thermotoga, Acinetobacter, Thermodesulfobacterium* species was the largest bacteria group in the digested effluents at 2-, 10- and 15-day, with greatest proportion at 15-day HRT. The predominant methanogens at the longer HRT of 10- and 15-day were still the acetoclastic *Methanosarcina thermophila* as observed in the low-, medium- and high-strength digested effluents at the shorter HRT of 2-day.

7.3.2. Thermophilic (55^{o}C) and mesophilic (37^{o}C) anaerobic single-stage digestion of high-strength piggery wastewater at 15-day HRT

For comparison purposes, data from the first-stage thermophilic reactor experiment on the undiluted high-strength piggery wastewater at 15-day HRT were included in all the Tables and Figures below alongside the mesophilic experimental data at 15-day HRT.

pH, alkalinity, ammonium-nitrogen and free ammonia

Table 7.8 gives the pH, total alkalinity, ionised ammonium-nitrogen and unionised dissolved free ammonia concentrations in the piggery influents and effluents of the thermophilic and mesophilic reactors at 15-day hydraulic retention times.

Table 7.8. pH, total alkalinity, ammonium-nitrogen and free ammonia concentrations of the piggery influents and effluents at thermophilic (T) and mesophilic (M) temperatures

Undiluted piggery wastewater	pH	Total alkalinity (mg CaCO3/L)	NH_4^+-N (mg/L)	Free NH_3-N (mg/L)
Influent to T-reactor	7.7 (0.1)	4550 (0)	1977 (21)	10 (1)
T-effluent	8.4 (0.1)	5894 (8)	2083 (42)	1026 (74)
Influent to M-reactor	7.5 (0.1)	4483 (76)	1986 (66)	7 (1)
M-effluent	8.0 (0.1)	5800 (0)	2119 (84)	234 (45)

Data are mean values (± standard deviation)

As shown in Table 7.8, following thermophilic (55^{o}C) and mesophilic (37^{o}C) digestion, pH and alkalinity of the effluents increased due to the release of more ammonia-nitrogen from the degradation of nitrogenous urea and protein materials.

Free ammonia concentration was substantially less in the mesophilic effluent compared to the thermophlic effluent. This was attributed largely to its lower reactor

temperature as pH sensitivity at a higher effluent pH of 8.4 as the thermophilic effluent resulted in a free ammonia concentration of 512 ± 20 mg N/L which was still significantly less than the level present in the thermophilic effluent (1026 ± 74 mg N/L).

Total and volatile suspended solids (TSS and VSS)

Table 7.9 gives the total solids (TS), volatile solids (TS), total suspended solids (TSS) and volatile suspended solids (VSS) data before and after thermophilic and mesophilic anaerobic treatments while Figure 7.10 illustrates the amount of solids removed.

Table 7.9. Solids concentrations in the undiluted influents and digested effluents at thermophilic (T) and mesophilic (M) temperatures

Undiluted piggery wastewater	TS (g/L)	VS (g/L)	TSS (g/L)	VSS (g/L)
Influent to T-reactor	8.1 (0.2)	5.0 (0.2)	3.4 (0.1)	3.0 (0.1)
T-effluent	7.5 (0.2)	4.3 (0.2)	2.7 (0.2)	1.9 (0.2)
Influent to M-reactor	8.0 (0.3)	5.0 (0.2)	3.1 (0.2)	2.9 (0.2)
M-effluent	6.6 (0.2)	3.6 (0.1)	2.4 (0.2)	1.9 (0.2)

Data are mean values (± standard deviation)

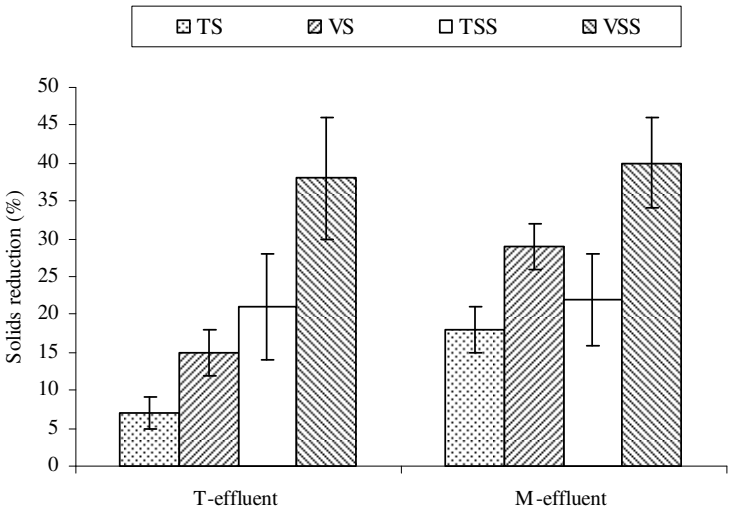


Figure 7.10. Solids reductions of thermophilic (T) and mesophilic (M) digested effluents at 15-d HRT (error bars indicate standard deviations)

Reductions of total and volatile solids were significantly ($p<0.05$) higher at mesophilic (37^{o}C) than at thermophilic temperature (55^{o}C). However, no significant differences in the reductions of total suspended solids (TSS) and volatile suspended solids (VSS) were observed between mesophilic and thermophilic reactor. The data implied that hydrolysis of the influent particulate organic matter was comparable between thermophilic and mesophic reactors which was in contrast to the calculated extent of net hydrolysis given in Table 7.13.

COD, volatile fatty acids and biogas

Table 7.10 gives the COD data of the piggery influents and digested effluents of the thermophilic and mesophilic reactors. The amount of COD removed from the digested effluents after anaerobic treatment was graphically presented in Figure 7.11.

Table 7.10. Chemical oxygen demand (total and soluble) and total VFA concentrations of the undiluted piggery influents and digested effluents at thermophilic (T) and mesophilic (M) temperatures

Undiluted piggery wastewater	Total COD (mg/L)	Soluble COD (mg/L)	Total VFA (mg COD/L)
Influent to T-reactor	11001 (562)	7270 (355)	5600 (304)
T-effluent	7151 (509)	4937 (405)	3026 (265)
Influent to M-reactor	11207 (127)	6848 (182)	6634 (142)
M-effluent	5640 (264)	2777 (212)	1046 (99)

Data are mean values (± standard deviation)

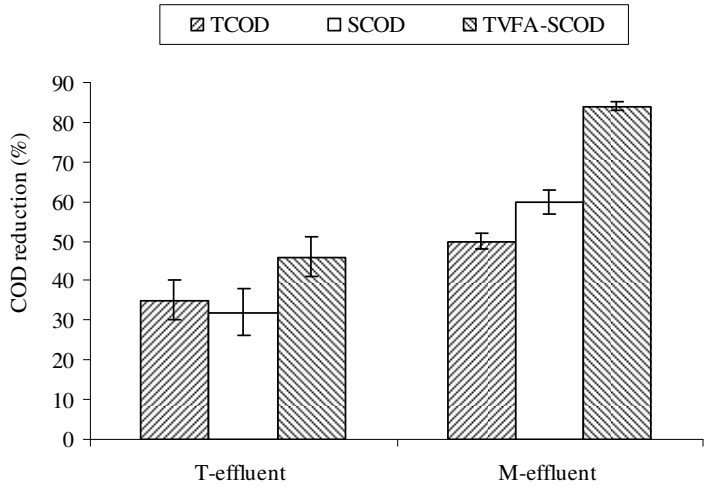


Figure 7.11. COD (total and soluble) and total VFA reductions of the thermophilic (T) and mesophilic (M) digested effluents at 15-d HRT (error bars indicate standard deviations)

Reductions of COD (total and soluble) and total VFA-COD were all significantly ($p<0.05$) higher at mesophilic (37^{o}C) than at thermophilic temperature (55^{o}C) which were in agreement with the total and volatile solids reductions (Figure 7.10).

Table 7.11 gives the volatile fatty acid components in the thermophilic and mesophilic digested effluents. The amount of VFA removal is graphically presented in Figure 7.12.

Table 7.11. Volatile fatty acid concentrations in the influents and digested effluents of the thermophilic (T) and mesophilic (M) reactors at 15-day HRT

Undiluted piggery wastewater	Acetate (mg COD/L)	Propionate (mg COD/L)	i-butyrate (mg COD/L)	n-butyrate (mg COD/L)	i-valerate (mg COD/L)	n-valerate (mg COD/L)	Caproate (mg COD/L)
Influent to T-reactor	2054 (87)	1334 (56)	451 (35)	498 (40)	698 (59)	431 (22)	134 (6)
T-effluent	631 (191)	2060 (79)	65 (18)	19 (19)	139 (41)	64 (21)	48 (3)
Influent to M-reactor	2548 (58)	1609 (37)	587 (13)	371 (15)	941 (21)	473 (23)	105 (3)
M-effluent	825 (81)	88 (13)	17 (7)	28 (18)	22 (2)	44 (21)	23 (10)

Data are mean values (± standard deviation)

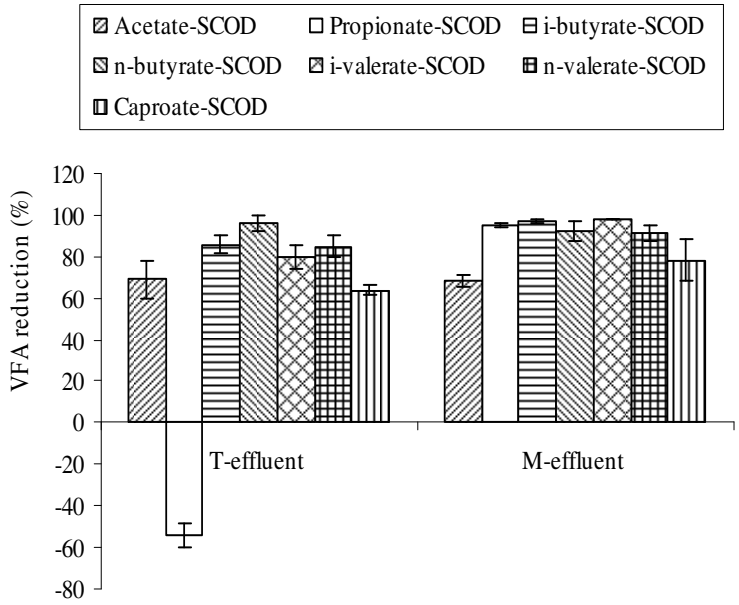


Figure 7.12. Percentage removal of VFA-COD in the thermophilic (T) and mesophilic (M) digested effluents at 15-d HRT (error bars indicate standard deviations)

Significant VFA reductions in acetate, i- & n-butyrate, i- & n-valerate and caproate were observed in the thermophilic and mesophilic digested effluent. However,

propionate concentration was greatly elevated in the thermophilic effluent in contrast to the substantially reduced concentration in the mesophilic effluent. This suggested that the reactor condition at mesophilic temperature was thermodynamically more favourable for acetogenic degradation of propionate than at thermophilic temperature.

Figure 7.13 illustrates the relationships between free ammonia concentrations and the amount of acetate degraded as well as propionate degraded as a function of reactor temperature. An inverse relationship was observed between propionate degradation and free ammonia concentration. However, no relationship existed between acetate degradation and free ammonia concentration.

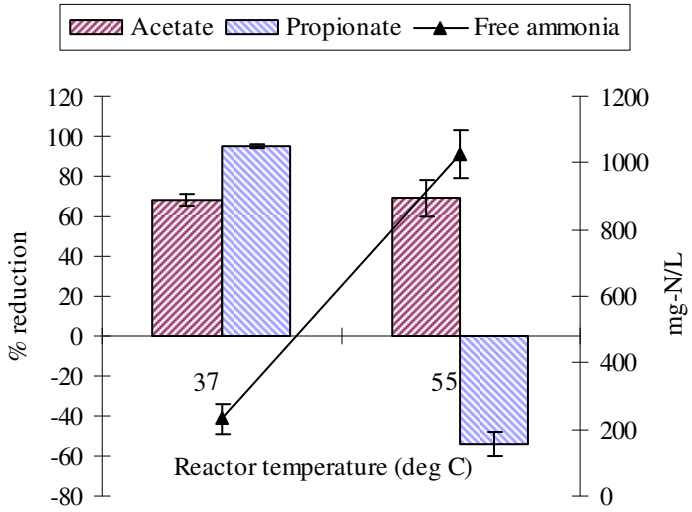


Figure 7.13. Relationships between acetate, propionate and free ammonia concentrations in the mesophilic (37^{o}C) and thermophilic (55^{o}C) digested effluents (error bars indicate standard deviations)

Table 7.12 gives the actual specific methane yields of the thermophilic and mesophilic reactors in terms of total TVFA-SCOD removed. Statistical t-test analysis ($p<0.05$) of the data indicated the thermophilic reactor produced significantly higher specific methane yield than the mesophilic reactor.

Table 7.12. Biogas composition and methane yields from the thermophilic (55°C) and mesophilic (37°C) reactors at 15-day HRT

Reactor	Organic loading rate (g TCOD fed/L/d)	Total VFA-SCOD removal rate (g COD/L/d)	% methane	% carbon dioxide	Actual specific methane yield at stp (L/g COD removed)
55°C	0.7334	0.172 (0.018)	71 (1)	24 (1)	0.322 (0.034)
37°C	0.7471	0.373 (0.007)	76 (1)	17 (1)	0.176 (0.005)

Data are mean values of eight measurements (± standard deviation)

The substantially lower specific methane yield from the mesophilic reactor was rather surprising as it produced substantially higher COD and total VFA reductions than the thermophilic reactor (Figure 7.11). Possible causes of the low specific methane yield at mesophilic temperature are given in section 6.4 of Chapter 6 and further elaboration can be found in the following section 7.4.2.

COD material balance

Figure 7.14 shows the COD material balance constructed from the COD components present in the undiluted piggery influents and digested effluents from the thermophic (T) and mesophilic (M) reactors relative to their respective influent TCOD concentrations. Although degradation was noted to have occurred during storage, it was not significant in terms of comparing degradation performace.

Apart from the doubtful high proportion of the other SCOD fraction in the thermophilic reactor influent (section 7.3.1), one particularly visible difference between the COD material balance of thermophilic and mesophilic digested effluents is their proportions of propionate fraction. Thermophilic digested effluent had higher proportion of propionate fraction (19%) than its influent (12%) whereas the mesophilic digested effluent had substantially lower proportion of propionate fraction (0.8%) than its influent (14%).

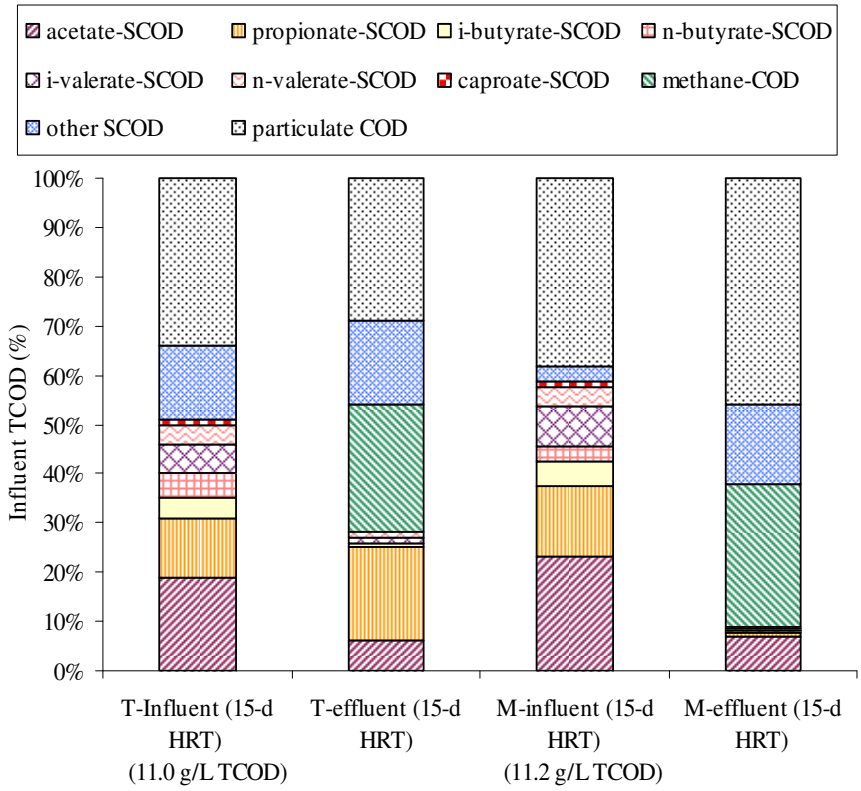


Figure 7.14. COD material balance of the undiluted piggery influents, thermophilic (T) and mesophilic (M) digested effluents at 15-day HRT

The COD material balance of thermophilic digested effluent shows 29% of the influent organic matter existed as non-biodegradable particulate organic matter, while propionate (19%) and other non-VFA soluble organics (17%) formed the two largest groups of undigested soluble organic matter. In the case of the COD material balance of mesophilic digested effluent, 46% of the influent organic matter existed as non-biodegradable particulate organic matter, while propionate the other non-VFA soluble organics (16%) made up the largest group of undigested soluble organic matter.

Reactor performance in the conversion of organics

Table 7.13 compares the digestion performance of the thermophilic and mesophilic anaerobic reactors at 15-day HRT. The data show that while small additional net hydrolysis of the influent particulate organic matter and net acidification of the soluble organics occurred in the thermophilic reactors, there was no further net hydrolysis and acidification occurring in the mesophilic reactor.

Table 7.13. Digestion performance data of the thermophilic (T) and mesophilic (M) reactors at 15-day HRT

Parameter HRT (days)	Unit	T-Effluent 15	M-Effluent 15
Extent of net hydrolysis	% TCOD fed	5 (3)	0
Extent of net acidification	% TCOD fed	2 (2)	0
Extent of methanogenesis	% TCOD fed	26 (1)	28 (1)

Data are mean values (± standard deviation)

The extent of organics conversion to methane was considered low at below 30% by both the thermophilic and mesophilic single-stage anaerobic reactors.

Anaerobic microbial populations

Table 7.14 gives the bacteria and methanogen populations enumerated by molecular FISH method in the undiluted piggery influents and digested effluents from the thermophilic and mesophilic reactors.

Table 7.14. Quantification of anaerobic thermophilic (T) and mesophilic (M) microorganisms by domain oligonucleotide FISH probes of ARC-915 for *archaea* and EUBMIX for *bacteria*

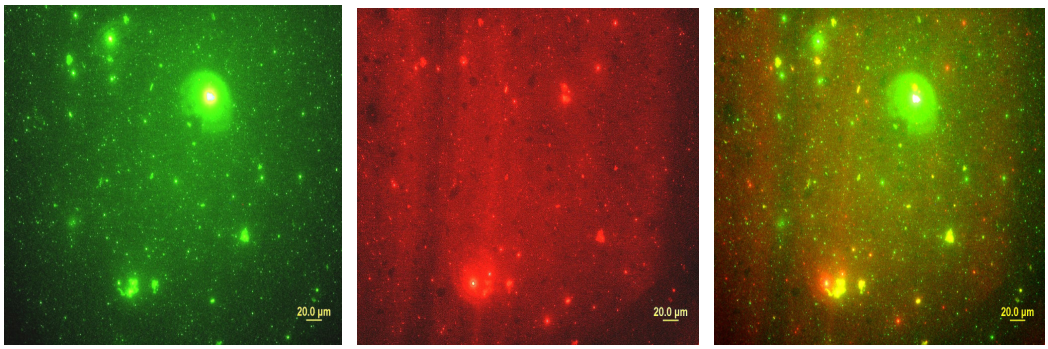
Undiluted piggery wastewater	*Bacteria* (cells/ml)	*Archaea* (cells/ml)
Influent to T-reactor	$(8.42 \pm 1.47) \times 10^8$	$(1.51 \pm 0.45) \times 10^9$
T-effluent	$(1.57 \pm 0.21) \times 10^9$	$(2.46 \pm 0.23) \times 10^9$
Influent to M-reactor	$(8.86 \pm 0.24) \times 10^8$	$(1.37 \pm 0.10) \times 10^9$
M-effluent	$(1.78 \pm 0.03) \times 10^9$	$(2.32 \pm 0.24) \times 10^9$

Data are mean values of replicate measurements (± standard deviation)

FISH results show the viable *archaea* levels were comparable between the thermophilic and mesophilic digested effluents while the bacteria level was slightly higher in the mesophilic effluent. As T-RFLP microbial profiling analysis was not determined on the mesophilic digested effluent, comparisons of the diversities of bacteria and methanogens betwen thermophilic and mesophilic effluents were precluded.

Figures 7.15 (a), (b) and (c) show the fluorescent images of the *archaea*, total *bacteria* (hydrolytic, acidogenic and acetogenic bacteria) and *archaea* plus *bacteria* in the mesophilic digested effluent at 15-day HRT.

(a) (b) (c)



Figures 7.15 (a), (b) and (c). Fluorescent images of mesophilic *archaea* (green), bacteria (red) and superimposed image of (a) and (b) at 15-d HRT

7.4. DISCUSSION

7.4.1. Performance comparisons of the thermophilic anaerobic reactors treating undiluted piggery wastewater at 2-, 10- and 15-day HRT

The observed inconsistencies in the solids and COD reduction trends at the three HRTs can be attributed to a combination of sample variability and measurement errors incurred particularly in the solids determination. Unlike the COD and VFA analysis, solids analysis involved multi-steps sample handling procedures such as weighing plus filtering (extra steps for TSS and VSS), weighing, drying, reweighing, combustion and reweighing (APHA Standard Method 2540 B). By taking these factors into consideration, it was decided that the COD and VFA removal data were considered more reliable than the solid removal data for assessing the reactor performance.

Despite the gradual increase in free ammonia concentration with increasing HRT, the strong positive correlations observed between free ammonia concentrations and the amount of acetate degraded (R^2 = 0.9451) and between total VFA reductions and specific methane yields (R^2 = 0.9873) demonstrated that the acetoclastic *Methanosarcina thermophila* which were identified to be the predominant

methanogens were adaptable to the effluent high free ammonia with increased HRT. The result concurred with previous studies that reported the ability of methanogenic cultures to adapt to increasing ammonia levels with increasing substrate loading and hydraulic retention time (Angelidaki and Ahring, 1994; Braun *et al*., 1981).

High hydrogen concentration greater than 10^{-4} atm (Leybo *et al*., 2006) and high acetate concentrations greater than 1mM (Leven *et al*., 2007; Yu *et al*., 2005; Diaz *et al*., 2003; Schmidt *et al*., 2000; section 2.2.4 in Chapter 2) are found to favour the growth of acetoclastic *Methanosarcina thermophila*. It is also found to dominate in swine and cattle manure digesters which typically contained high levels of ammonia (NH_3), hydrogen sulfide (H_2S) and volatile fatty acids (VFA) whereas filamentous *Methanosaetaceae* dominated in sewage sludge digesters with low levels of NH_3 and VFA (Demirel and Scherer, 2008; Karakashev *et al*., 2005; Mladenovska *et al.*, 2003; Schmidt *et al*., 2000). The findings of *Methanosarcina thermophila* being the predominant methanogen in the thermophilic digested piggery effluents of 10- and 15-day HRT that contained high acetate levels of 10mM (592 mg/L) and 13mM (787 mg/L) respectively were in agreement with these previous studies.

The accumulation of propionate particularly at longer HRT of 10- and 15-day was the culmination of greater amount of n-valerate being degraded to propionate, acetate and hydrogen, in conjunction with increased hydrogen being released from more degradation of n-butyrate and i-valerate to acetate and hydrogen (Figure 7.13). The degradation reactions of volatile fatty acids and the fermentation products formed are given in Table 2.2 of Chapter 2. High hydrogen concentration or hydrogen partial pressure in excess of 10^{-4} atm are well known to inhibit the propionate-degrading bacteria from oxidizing propionate to acetate, hydrogen and carbon dioxide, thus leading to propionate accumulation (section 2.5.7 of Chapter 2). Thus, efficient anaerobic oxidation of propionate requires the obligate syntrophic consortia of acetogenic proton and bicarbonate-reducing bacteria and hydrogen-utilising methanogenic *archaea* or SRB to be in close proximity to facilitate efficient inter-species hydrogen or formate transfer to occur in order to maintain a low hydrogen partial pressure below 10^{-4} atm (Speece *et al.*, 2006; de Bok *et al*., 2004). As the GC used for biogas compositional analysis in these experiments could not measure hydrogen concentration below 1% due to its high detection limit, no concrete

hydrogen data was available to directly link it to the propionate build-up observed in these experiments.

Nevertheless, the elevated propionate concentrations implied that hydrogen-utilising microorganisms were inhibited by the high ammonia concentrations in the wastewaters. Wiegant and Zeeman (1986) found that when the hydrogen-utilising methanogens were inhibited by high ammonia concentration, propionate accumulated. It is therefore reasonable to assume that the inhibition of hydrogen-utilising microorganisms would led to the build-up of hydrogen partial pressure which in turn inhibited the propionate-degrading bacteria from converting propionate to acetate, hydrogen and carbon dioxide, thus resulting in the accumulation of propionate (Table 2.2 in Chapter 2). The negative correlation coefficient value (R^2 = -0.7469) obtained between free ammonia concentration and the amount of propionate degraded in the digested piggery effluent (Figure 7.4) demonstrated that the propionate-degrading acetogenic bacteria were sensitive to the increased free ammonia concentrations with increasing HRT. Previous studies have reported that the propionate degraders are the slowest growing and most sensitive VFA-degrading microoganisms in the anaerobic digestion system (Nielsen *et al*., 2007; Ahring *et al.*, 2001; Ozturk, 1991). On this basis, Nielson *et al.* (2007) suggested that propionate was far more reliable key parameter than methane production, pH or propionate:acetate ratio as indicator of process imbalance in biogas plants treating complex organic wastes as well as for the regulation and optimization of the biogas process.

The unaffected high butyrate and valerate degradation at the longer HRT of 10- and 15-day (Figure 7.3) demonstrated the higher tolerance of butyrate-degrading acetogenic bacteria to hydrogen concentration as opposed to the propionate-degrading bacteria. Similar finding of high butyrate degradation while propionate degradation was inhibited by free ammonia concentration above 200 mg/L has been reported in previous study (Calli *et al.* 2005). For propionate degradation to proceed, up to 5 to 6 times lower hydrogen concentration is required than butyrate degradation (Pind *et al.*, 2003).

Total ammonia, in particular the free ammonia concentration is well documented in the literature to be the main cause of low methane yield from livestock wastes,

particularly at thermophilic conditions (Hansen *et al*., 1999, 1998; Angelidaki and Ahring, 1994; Wiegant and Zeeman, 1986; Braun *et al*., 1981). The total ammonium-nitrogen concentrations of 2.1 g-N/L and free ammonia concentrations of around 1 g-N/L at the longer HRT of 10- and 15-day were high compared to the literature values of 1.5-3.0 g-N/L total ammonia and 0.56-0.7 g-N/L free ammonia respectively. These values were reported to be inhibitory to methanogenesis at pH 7.4-7.9 (Sung and Liu, 2003; Angelidaki and Ahring, 1994; Hiroshima 1983; Braun *et al*., 1981). Acclimatisation of methanogenic biomass to increased ammonia levels and reduction of free ammonia concentrations through dilution and temperature reduction are two means to lessen the impact of ammonia inhibition on the mixed anaerobic cultures.

The adaptability of the acetoclastic methanogens to high free ammonia concentration was demonstrated in the gradual increase in specific methane yields with increasing HRT from 2- to 15-day. The methane yield (26%) at the longer HRT of 15-day HRT was however considered low as 20% of the piggery influent organics remained as undigested particulate organics while propionate (19%) and other non-VFA soluble organic matter (17%) made up the two largest groups of the unconverted soluble organic fraction. These undigested organics represented a big proportion of residual biogas potential (Angelidaki *et al*., 2006) in the digested effluents. Possible organic components that could be present in the other non-VFA soluble fraction include unused substrates such as amino acids, long-chain fatty acids and other metabolic products such as dead microbial cells and other organic acids and solvents as mentioned in section 5.4 of Chapter 5.

7.4.2. Performance comparisons of the thermophilic and mesophilic single- stage anaerobic reactors treating undiluted piggery wastewater at 15-day HRT

The substantially higher level of total VFAs, in particular propionate at thermophilic temperature compared to mesophilic temperature in this experiment was consistent with the findings of other researchers (Speece *et al*., 2006; Aitken *et al*., 2005a; Song *et al*., 2004; Gavala *et al*., 2003b; Kim *et al*., 2002; Angelidaki and Ahring, 1994; Hashimoto, 1983). The observed inverse relationship between the amount of propionate degraded and free ammonia concentrations in the thermophilic and

mesophilic digested effluents (Figure 7.13) linked the propionate accumulation in the thermophilic effluent to its high free ammonia concentration. It has been recognized that there are three possible pathways of propionate degradation depending on sulfate concentration in the wastewaters: 1) degradation by propionate-utilising sulfate-reducing bacteria (SRB) such as *Desulfobulbus*; 2) syntrophic degradation by propionate-degrading acetogenic bacteria in association with hydrogenotrophic SRB; and 3) syntrophic degradation by propionate-degrading acetogenic bacteria in association with hydrogenotrophic methanogens (Ariesyady *et al*., 2007b; section 2.2 in Chapter 2).

Thus, the poor removal of propionate indicated that the obligate syntrophic consortia of propionate-degrading acetogenic bacteria and hydrogenotrophic methanogens and /or hydrogenotrophic SRB were being severely inhibited in the thermophilic anaerobic reactor. Acetate degradation was however not affected by the high free ammonia level in the thermophilic effluent as shown by the lack of relationship between acetate degradation and free ammonia concentrations in the thermophilic and mesophilic effluents. The observation suggested that the thermophilic acetoclastic methanogens were more resilient to product inhibition than the syntrophic consortia of propionate-degrading bacteria and hydrogenotrophic methanogens or SRB.

As propionate degradation strongly depends on the maintenance of low hydrogen partial pressure or concentration, the elevated propionate concentration of the thermophilic effluent indicated that the syntrophic hydrogenotrophic methanogens and/or SRB were inhibited by the high free ammonia concentration which in turn impacted negatively on the propionate-degrading acetogenic bacteria. Free ammonia is widely known as the active component of the total ammonia that causes ammonia inhibition as the unionised molecule can freely penetrate the microbial cell membrane and disrupts the intracellular metabolism (Gerardi, 2006). In this experiment, although the total ammonium-nitrogen concentrations in the thermophilic and mesophilic digested effluent were both high at 2.1 g-N/L, the free ammonia concentration in the thermophilic effluent was much higher (1.0 g-N/L) than in the mesophilic effluent (0.23 g-N/L) due largely to the higher reactor temperature.

The high free ammonia level of the thermophilic digested effluent was above the inhibitory value of 700 mg-N/L at pH 7.6-7.9 reported by Angelidaki and Ahring (1994) and also way above the inhibitory value of 560-568 mg-N/L reported by Gallert and Winter (1997) that caused 50% inhibition of the methanogenesis at pH 7.6. Hansen *et al.* (1998) found hydrogen-utilising methanogens in particular to be inhibited by free ammonia concentration higher than 1.1 g-N/L. Wiegant and Zeeman (1986) noted that the hydrogen-utilising methanogens were more severely affected by high ammonia concentration than the acetoclastic methanogens. They suggest that the elevated propionate observed at thermophilic conditions was due to the propionate-degrading bacteria being inhibited by the high hydrogen concentration or partial pressure. The latter was the outcome of the hydrogen-utilising methanogens being inhibited by the high ammonia level.

Speece and co-workers (2006) demonstrated that the poor thermophilic effluent quality could be solved through the use of alternative process configuration such as a non-mixed thermophilic CSTR or immobilized methanogenic upflow anaerobic sludge blanket (UASB) reactor as part of a two-stage anaerobic system. These process configurations provide close microbial consortia proximity that enhances interspecies electron transfer of hydrogen and/or formate which was lacking in the conventional CSTR, besides retaining the biomass in the case of a UASB reactor. As a result, higher OLR and improved thermophilic effluent quality were achieved.

In the mesophilic digested effluent, the much lower free ammonia concentration which corresponded with the greatly reduced propionate concentration (Figure 7.13) demonstrated the beneficial effect of reduced temperature for the control of free ammonia and propionate concentrations. Although reductions of total VFA and soluble COD were much higher than the thermophilic effluent (Figure 7.11), the specific methane yield obtained was surprisingly less than the thermophilic reactor and much less than the theoretical specific methane yield (Table 7.12). Nonetheless, the comparative results were in accordance with Hashimoto (1983) but contrasted with van Velsen *et al.* (1979) who reported higher methane yield from mesophilic than thermophilic reactors.

One possible cause besides gas measurement errors for the observed lower specific methane yield at mesophilic temperature could be that there was greater microbial

competition from the higher diversity of non-methanogenic mesophilic bacterial trophic groups for common methanogenic substrates as opposed to at thermophilic temperature. Table 7.15 gives the known bacterial trophic groups that can compete with the syntrophic acetogenic and methanogenic *archaea* for VFAs and hydrogen substrates which serve as electron donors.

Table 7.15. Redox reactions of the competing anaerobic microorganisms

Substrate	Redox reactions	ΔG^o(kJ/mol)
Propionate	$CH_3CH_2COO^- + 0.75\ SO_4^{2-} \rightarrow CH_3COO^- + HCO_3^- + 0.75\ HS^- + 0.25\ H^+$ (by sulfate-reducing bacteria)	-37.7
Propionate	$CH_3CH_2COO^- + 1.75\ SO_4^{2-} \rightarrow HCO_3^- + 1.75\ HS^- + 0.5\ H^+ + 0.25\ OH^-$ (by sulphate-reducing bacteria)	-88.9
Propionate	$CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + 3H_2 + CO_2$ (by propionate-oxidizing acetogenic bacteria)	+76.1
Acetate	$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$ (by sulfate-reducing bacteria)	-47.6
Acetate	$CH_3COOH \rightarrow CH_4 + CO_2$ (by acetoclastic methanogens)	-31.0
Hydrogen	$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$ (by sulphate-reducing bacteria)	-38.1
Hydrogen	$4H_2 + SO_4^{2-} \rightarrow S^{2-} + 4\ H_2O$ (by sulfate-reducing bacteria)	-151.0
Hydrogen	$4H_2 + 2CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$ (by homoacetogens)	-95.0
Hydrogen	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ (by H_2–utilizing methanogens)	-131.0
Hydrogen	H_2 + AQDS → AH_2QDS (by humics-reducing bacteria)	-44.4

(Sources: van Haandel *et al*., 2006; Gallert and Winter, 2005; Cervantes *et al.*, 2000; Brock *et al*., 1994)

As indicated by the greater negative standard Gibbs free energy change ΔG^o, sulfate-reducing bacteria (SRB) can out-compete methanogens for the propionate, acetate and hydrogen in the anaerobic digester due to their higher affinity for these electron donors. *Desulfovibrio* sulphate-reducing bacteria had been identified by T-RFLP molecular profiling to be present in the undiluted piggery effluent in this experiment. It is thus possible that the complex microbial ecology and its dynamics could have a major influence on the performance of anaerobic digester.

As propionate is the most difficult VFA to degrade and can only be degraded at very low hydrogen partial pressure (<10^{-4} atm), the low effluent propionate concentration in the mesophilic anaerobic reactor was clear evidence that the thermodynamic conditions were highly favourable for the hydrogen-sensitive propionate-degrading acetogenic bacteria to efficiently convert the propionate to acetate. It is postulated that a combination of low free ammonia and total VFA concentrations as well as lower reactor temperature had created a favourable environment for the growth of the hydrogen-utilising microorganisms in particular. These microbes were able to efficiently remove the hydrogen released during VFA degradation, thus maintaining the hydrogen partial pressure at a very low level and facilitated enhanced propionate removal by the propionate-degrading bacteria.

Although the chemical quality of mesophilic digested effluent was superior to that of its thermophilic counterpart, numerous studies have demonstrated unanimously that anaerobic digestion of wastewaters and sewage sludge at mesophilic temperatures is inferior in pathogens reduction or disinfection than at thermophilic temperatures (Bajsa, unpublished; Salsali *et al.,* 2008; Gray *et al*., 2006; Bagge *et al*., 2005; Smith *et al*., 2005; Sahlström, 2003; Olsen and Larsen, 1987; Watanabe *et al*., 1997; Bendixen, 1994; Kearney *et al.*, 1993; Duarte *et al.*, 1992). It is likely that a combination of the effluent low levels of toxic free ammonia and total volatile fatty acids in conjunction with its lower reactor temperature had contributed to the widely acknowledged poor pathogens reduction by the mesophilic anaerobic reactor. It is reported that between the mesophilic temperature range of 30-40^{o}C, growth and survival of enteric bacteria is optimum (Smith *et al*., 2005) which may explain why microbial species diversity at mesophilic temperatures is so much greater than at thermophilic temperatures as observed in previous studies (Kobayashi *et al.*, 2008; Leven *et al*., 2007; Karakashev *et al*., 2005; Sekiguchi *et al*., 1998). The diverse and complex microbial dynamics could possibly have played a key role in the high propionate degradation (95%) observed in the mesophilic anaerobic reactor as opposed to its thermophilic counterpart.

7.5. CONCLUSION

The results from these studies showed that thermophilic single-stage anaerobic reactor operating at 15-day HRT converted significantly more influent volatile fatty acids to methane biogas than at 2- and 10-day HRT. It also had significantly higher specific methane yield based on TVFA-COD removed than mesophilic reactor at 15-day HRT. However, the methane yield was considered low in relation to the influent total organics or COD material balance. A large residual biogas potential still remained in the thermophilic digested effluent in the forms of particulate organics and unconverted soluble organics (other non-VFA organics and propionate). Although the chemical quality of the thermophilic effluent was inferior to that of the mesophilic effluent particularly with respect to its high levels of free ammonia, COD, total VFA and propionate, thermophilic anaerobic digestion at 55^{o}C is unversally known for its effectiveness in eliminating most pathogenic bacteria than mesophilic digestion at 35-37^{o}C.

CHAPTER 8
THERMOPHILIC ANAEROBIC BATCH VIAL EXPERIMENTS TO MITIGATE AMMONIA INHIBITION AND TO ENHANCE METHANE PRODUCTION FROM PIGGERY EFFLUENT

8.1. INTRODUCTION

Anaerobic digestion of livestock wastes including piggery is widely known to produce low methane yield due in part to microbial inhibition by the high ammonia released from the fermentation of urea and nitrogen-containing protein material (Hansen *et al*., 1998; Angelidaki and Ahring, 1994; Braun *et al*., 1981). Results presented in chapter seven of this thesis concurred with the universal findings. Means to reduce the ammonia inhibition on biogas production from livestock wastes have been extensively investigated by numerous researchers and their findings were comprehensively reviewed by Yadvika *et al*. (2004) and Chen *et al.* (2008). These included reducing the reactor effluent pH, diluting the digester feedwater, recirculating digested slurry to the reactor, process modification, gradual acclimatisation of the biomass with reduced organic loading rate and additions of inorganic and organic additives such as zeolite, activated carbon, clay and iron to adsorb the inhibitory compounds.

As an extension to these studies which investigated the effect of a single entity (pH, chemical or biological) on methane production, this chapter presents experimental findings from five sets of thermophilic (55^{o}C) batch vial experiments which investigated the effect of a single entity (pH, chemical or biological) and also the combined effects of two entities such as pH plus chemicals or pH plus biological supplements in enhancing methane production from thermophilic anaerobic digestion of piggery waterwater.

The first set of experiment investigated the effect of pH reduction on methane production from the thermophilic digested piggery effluent at its initial pH of 8.2 to pH 6.5. As thermophilic conditions and pH above 7.4 increase the free ammonia concentration and inhibit the methanogenesis process (Gallert and Winter, 1997;

Angelidaki and Ahring, 1994), the aim of this experiment was to establish the optimum wastewater pH that would enhance biodegradation of the organic carbon compounds to methane biogas.

The second and third sets of batch vial experiments investigated the effects of supplementing two types of biomass to the digested piggery wastewater on methane production from the thermophilic anaerobic digestion of piggery wastewater. As the conventional continuously-stirred tank anaerobic reactor (CSTR) configuration does not incorporate devices that retain the microbes which are washed away during the reactor operation, supplementing biomass to the digested effluent was thus akin to recirculating the digested slurry back to the reactor to boost the microbial population. Inoculation of microbes from other sources has also been found to enhance biogas production by stimulating the microbial activity and/or the production of particular enzymes which were lacking (Bagi *et al*., 2007; Yadvika *et al*., 2004; Angelidaki and Ahring, 2000). Two types of biomass were used in these comparative experiments: 1) piggery biomass obtained from the digested piggery effluent of the lab-scale thermophilic (55^{o}C) anaerobic reactor; and 2) biomass obtained from a local aerobic-anaerobic municipal solid waste treatment plant (DiCOM). The objectives of these series of experiments were: 1) to examine and compare the enhancement effect on methane production of different amounts of biomass (piggery or DiCOM) supplements to the pH-unadjusted piggery wastewater against the pH-unadjusted control; 2) to examine and compare the enhancement effect on methane production of different amount of biomass (piggery or DiCOM) supplements to the pH-reduced piggery wastewater against the pH-reduced control; and 3) to compare the methane enhancement effects of piggery biomass supplements with DiCOM biomass supplements on the pH-unadjusted and pH-reduced piggery wastewaters.

The fourth set of batch vial experiment investigated the methane enhancement effect of natural zeolite at various concentrations on thermophilic anaerobic digestion of pH-unadjusted and pH-reduced piggery wastewaters. Natural zeolite has been demonstrated by several researchers to improve organic matter degradation and methane production in the anaerobic digestion of piggery manure at mesophilic (Montalvo *et al*., 2006, Milan *et al*., 2001; 2003; Sánchez *et al*., 1995) and thermophilic temperatures (Kotsopoulos *et al*., 2008) as well as sludge at mesophilic

temperature (Tada *et al*., 2005). Its capabilities to immobilise microorganisms as well as remove ammonia and ammonium ions through adsorption and ion-exchange with the zeolite inorganic minerals were cited as some of the reasons for the process improvement. In this experiment, Castle Mountain zeolites which are naturally occurring Clinoptilolite minerals (85%) found in New South Wales (Australia) were used. The zeolites consist largely of covalently-bonded aluminosilicates with interconnected cavities that are occupied by cations (calcium, magnesium, potassium, sodium) and water molecules which allow the zeolites to trap and exchange ions with the surrounding medium (www.castlemountainzeolites.com.au). The objectives of this experiment were: 1) to examine the methane enhancement effects of zeolite at various concentrations on the digested piggery wastewater with and without pH reduction to pH 6.5, and 2) to compare the methane enhancement effect of zeolite on pH-unadjusted piggery wastewater with the pH-reduced wastewater.

The fifth set of batch vial experiment investigated the methane enhancement effect of humic acid at various concentrations on thermophilic anaerobic digestion of pH-unadjusted and pH-reduced piggery wastewaters. Humic substances (humus) are heterogeneous high molecular weight organic matter which are ubiquitous in terrestrial and aquatic environments (Scott *et al*., 1998; Lovley *et al*., 1996). They are formed from the decomposition of plants, animals and microbial cells and accumulated in soils, water and sediments over time. Although humus is considered inert by itself to microbial degradation, it has been shown to possess electron-accepting capability in the anaerobic oxidation of organic carbon compounds and hydrogen. It has also been shown to shuttle electrons between humics-reducing microorganisms and humics-oxidising microorganisms or between humics-reducing microorganisms and insoluble metal oxides such as Fe (III) and Mn (IV) oxides as electron acceptors in anaerobic environments (Stams *et al*., 2006; Lovley *et al*., 1996). The ability of humic substances to mediate abiotic electron transfer has been attributed to the quinone moieties within humic substances that serve as electron-acceptors while the resultant reduced hydroquinone serve as electron-donors in microbial humics respiration (Scott *et al*., 1998, Lovley *et al*., 1999). Humic acid is the fraction of humus generally containing the highest concentration of quinones and it has been used in studies investigating the microbial reduction of humus (Cervantes *et al*., 2002, 2000; Benz *et al*., 1998; Lovley *et al*., 1996). The objectives of this

experiment were: 1) to examine the methane enhancement effects of humic acid at various concentrations on the piggery wastewater with and without pH-reduction to pH 6.5; and 2) to compare the methane enhancement effect of humic acid on pH-unadjusted piggery wastewater with the pH-reduced wastewater.

8.2. MATERIALS AND METHODS

8.2.1. Effect of pH on methane production from thermophilic batch digestion of piggery wastewater

Digested piggery effluent collected during the lab-scale thermophilic anaerobic reactor experiment at 10-day hydraulic retention time was used to provide the mixed anaerobic microorganisms. To ensure there were adequate degradable volatile fatty acids as food source for the natural microorganisms in the digested effluent, 10% v/v chilled raw piggery wastewater was added to the digested effluent. The piggery mixture was then sub-divided into four test portions. One test portion served as the control with no pH-adjustment while the other three test portions had their pH adjusted from pH 8.3 to 7.5, 7.0 and 6.5 with concentrated hydrochloric acid. The initial test samples at time zero were taken for biological (real-time PCR) and chemical (pH, total and soluble COD, VFA and ammonia-nitrogen) analysis. Following this procedure, 50 ml of each test sample was dispensed into two replicate serum vials of 120 ml volume, capped with butyl rubber stoppers and crimped tightly with aluminium seals.

Prior to incubating the vials in the shaking water bath, the vials' headspace was purged with high purity nitrogen to displace the air. After 20 minutes of incubation at 55^{o}C, the vials were degassed with syringe-needle to commence the 10-day batch anaerobic digestion experiment. Biogas volume was measured daily with plunger displacement of an air-tight glass syringe attached with metal hub needle whilst biogas composition was analysed daily for methane, carbon dioxide and hydrogen using Varian gas chromatograph. From the biogas volume and methane concentration measured, cumulative methane production was calculated for each vial. At the conclusion of the experiment, the vials were opened to allow samples to be

taken for final pH, total and soluble COD, VFA, ammonia-nitrogen and real-time PCR analysis.

8.2.2. Effect of biomass supplements on methane production from thermophilic batch anaerobic digestion of pH-unadjusted and pH-reduced piggery effluent

Two sources of biomass were tested for their digestion efficiency in this experiment: 1) piggery biomass of the digested effluent from the lab-scale thermophilic anaerobic reactor operating at 10-day HRT; and 2) DiCOM biomass from a local patented aerobic-anaerobic pilot digester that treated municipal solid wastes. The latter biomass was anticipated to be phylogenetically different and perhaps unique in their biochemistry due to the combined aerobic-anaerobic operating cycles in comparison to the anaerobic piggery biomass from a conventional anaerobic reactor. The piggery biomass in the digested effluent was concentrated by gravity settling and centrifuging at 4000 g for 5 minutes prior to its use. Samples of the DiCOM and piggery biomass were taken for TS and VS determinations. To ensure there were adequate degradable volatile fatty acids as food source for the additional anaerobic microorganisms added, a larger volume of 30% v/v as opposed to 10% v/v chilled raw piggery wastewater in the earlier experiment was provided to the digested effluent.

The digested effluent was then sub-divided into two test portions. One test portion served as the control with no pH adjustment while the other test portion had its pH adjusted with concentrated hydrochloric acid from pH 8.4 to the optimum pH 6.5 established in the earlier batch vial experiment on pH effect. Following this procedure, 10% and 19% v/v piggery biomass and DiCOM biomass were added separately to each of the prepared pH-unadjusted and pH-reduced digested piggery effluents. Initial test samples at time zero were taken for biological (real-time PCR) and chemical (pH, total and soluble COD, VFA and ammonia-nitrogen) analysis. Aliquots of 50 ml from the pH-unadjusted and pH-reduced digested piggery effluents with and without biomass additions were dispensed into two replicate serum vials of 120 ml volume. The vials were capped with butyl rubber stoppers and crimped tightly with aluminium seals. The same procedures of incubation and testing of biogas and mixed liquor as the earlier pH effect experiment in section 8.2.1 were adopted.

8.2.3. Effect of zeolite treatment on methane production from thermophilic batch anaerobic digestion of pH-unadjusted and pH-reduced piggery effluent

The same test method and analysis as in section 8.2.2 was adopted with the exception of the biomass being replaced by Castle Mountain natural zeolite (particle size less than 1 mm). The zeolite was added to both the pH-unadjusted and pH-reduced digested piggery effluents to give final concentrations of 0 (control), 10, 15 and 20 g/L.

8.2.4. Effect of humic acid supplements on methane production from thermophilic batch anaerobic digestion of pH-unadjusted and pH-reduced piggery effluent

The same test method as in section 8.2.3 was adopted with the exception of zeolite being replaced by humic acid (Sigma-Aldrich, technical grade) to give final concentrations of 0 (control), 1, 5, 10, 15 and 20 g/L. For the low concentrations of 1 and 5 g/L, only pH-reduced digested piggery effluent was tested following the unfavourable biogas production outcomes from the 10, 15 and 20 g/L humic acid at pH 8.1 (pH-unadjusted) and pH 6.5 (pH-reduced).

8.3. RESULTS

8.3.1. Effect of pH on methane production from thermophilic batch digestion of piggery effluent

Methane production

Figure 8.1 illustrates the effect of pH on cumulative methane production as a function of time and Figure 8.2 shows the changes in methane production rate over the 10-day digestion period. Reduction of the piggery effluent pH from 8.3 to 6.5 was observed to yield the greatest enhancement effect on methane production, followed by pH reduction to 7.5 and 7.0. At all four pHs, methane production rate was highest during the first 4 days of digestion but decreased thereafter due to substrate limitation setting in with the batch treatment mode. Despite the methane production rate at pH 6.5 dropping fastest in comparison to pH 8.3 (control), 7.5 and

7 as a result of its highest microbial activity, its methane production was highest at the end of the test period.

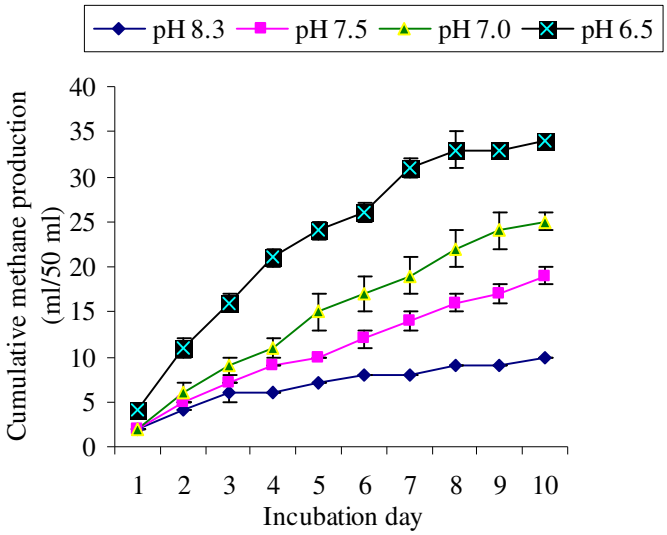


Figure 8.1. Effect of pH on methane production from thermophilic batch digestion of piggery wastewater (error bars indicate standard deviations)

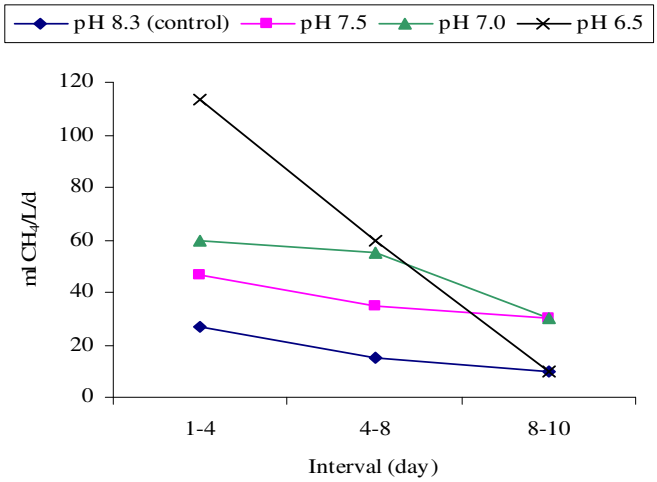


Figure 8.2. Changes in methane production rate with time during thermophilic batch digestion of piggery wastewater

Organic reduction (TCOD, SCOD and TVFA-SCOD)

Table 8.1 gives the COD (total and soluble) and total VFA concentrations while Figure 8.3 shows the percentage reduction data of total COD, soluble COD and total VFA-SCOD at the four wastewater pHs. The negative perceage reductions indicate

accumulation of the organics. As observed in the previous experiments, the higher SCOD values over TVFA-SCOD were due largely to the presence of non-VFA soluble components in the soluble fraction.

Table 8.1. Chemical oxygen demand (total and soluble) and total VFA of the thermophilic piggery wastewater at start and end of the batch serum vial digestion experiment

pH	Day	TCOD (mg/L)	SCOD (mg/L)	TVFA (mg COD/L)
8.3	0	7134 (0)	3138 (267)	1301 (157)
	10	6934 (947)	3605 (146)	2059 (26)
7.5	0	7455 (113)	3006 (240)	1373 (8)
	10	7235 (601)	3365 (219)	1551 (29)
7	0	7335 (284)	3025 (0)	1281 (28)
	10	6693 (154)	3167 (204)	1102 (77)
6.5	0	7327 (281)	3082 (187)	1175 (22)
	10	6132 (303)	2571 (93)	497 (19)

Data are mean values of replicates (± standard deviation)

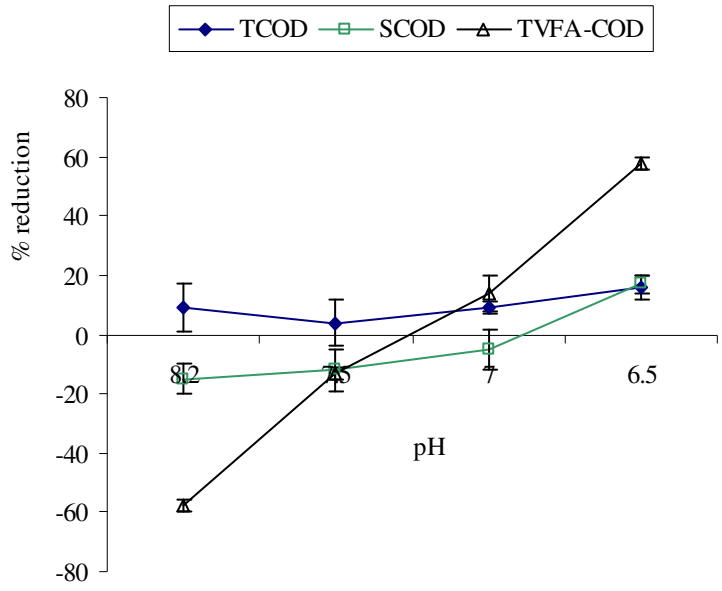


Figure 8.3. COD (total and soluble) and total TVFA-COD reductions as a function of thermophilic piggery wastewater pH after 10 days of batch digestion (error bars indicate standard deviations)

Figure 8.3 clearly shows pH 6.5 produced the highest enhancement effect on the reductions of total VFA and COD, followed by pH 7. The enhanced total VFA reduction with decreasing pH from 8.3 to 6.5 strongly correlated positively (R^2 = 0.9983) with enhanced methane production as illustrated in Figure 8.4. The small increased methane production observed at pH 8.3 (control) and pH 7.5 with no measurable TVFA or COD reduction after 10 days batch digestion could possibly

have come from the conversion of hydrogen and carbon dioxide to methane by the hydrogenotrophic methanogens.

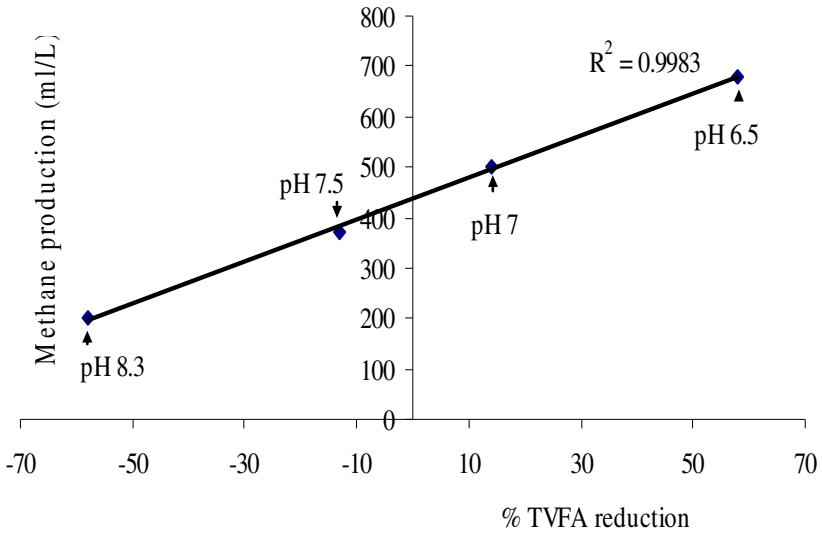


Figure 8.4. Relationship between total VFA-COD degraded and methane production at different wastewater pH after 10 days of batch digestion

Volatile fatty acid degradation

Table 8.2 gives the VFA components present in the soluble organic fraction and Figures 8.5 shows the percentage VFA degradation at the four wastewater pHs. Negative percentage degradation indicates VFA accumulation.

Without pH reduction, the piggery wastewater at pH 8.3 showed greatly elevated acetate and propionate concentrations at the end of the batch digestion. The accumulation of acetate and propionate was clear indication that the acetate-consuming methanogens and syntrophic propionate-degrading acetogenic bacteria-hydrogenotrophic methanogens were under stress in the environment that had become thermodynamically unfavourable. This was due to the formation of lower carbon number VFAs of acetate and propionate in addition to hydrogen from butyrate (i- and n-) and valerate (i- and n-) degradation (Table 2.2 in Chapter 2).

Table 8.2. Volatile fatty acid concentrations of the thermophilic piggery wastewater at the start and end of the test period

Initial pH	Day	Acetate (mg/L	Propionate (mg/L)	i-butyrate (mg/L)	n-butyrate (mg/L)	i-valerate (mg/L)	n-valerate (mg/L)	Caproate (mg/L)
8.3	0	370 (63)	355 (45)	41 (3)	32 (4)	72 (6)	29 (3)	14 (1)
	10	1159 (6)	464 (21)	15 (1)	7 (0)	27 (1)	0	13 (0)
7.5	0	410 (10)	372 (2)	36 (6)	34 (1)	75 (1)	31 (0)	13 (1)
	10	731 (27)	452 (27)	14 (4)	0	18 (4)	0	13 (1)
7	0	368 (23)	350 (8)	40 (2)	30 (1)	70 (2)	29 (1)	13 (1)
	10	399 (59)	401 (8)	11 (1)	0	12 (0)	0	12 (0)
6.5	0	335 (8)	322 (7)	40 (4)	27 (0)	62 (3)	28 (1)	12 (1)
	10	121 (0)	227 (14)	0	0	12 (1)	0	0

Data are mean values of replicates (± standard deviation)

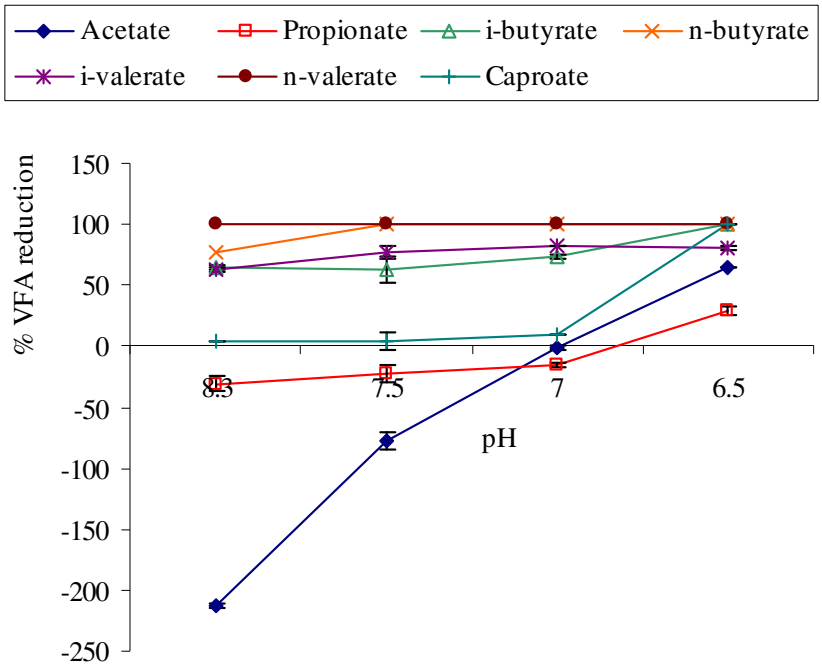


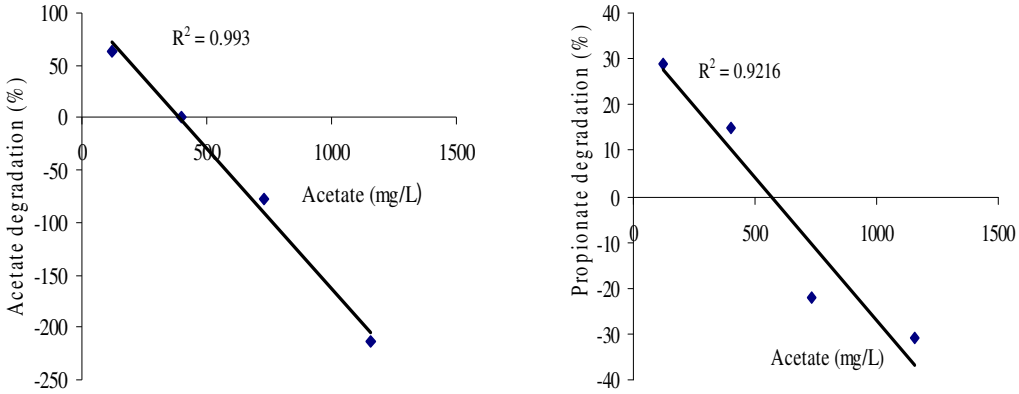
Figure 8.5. VFA degradation as a function of thermophilic piggery wastewater pH (error bars indicate standard deviations) after 10 days of batch digestion

Reducing the piggery wastewater pH from 8.3 to 7.5 and 7.0 resulted in substantial reductions of the elevated acetate although the final concentration was still elevated. Propionate reduction was minimal. Reducing the pH further to 6.5 further enhanced the degradation of acetate, propionate, i-butyrate and caproate (Figure 8.5). The elevated acetate and propionate concentrations at initial wastewater pH of 7, 7.5 and 8.3 which coincided with the degradation of butyrate (i- and n-) and valerate (i- and n) to acetate, propionate and hydrogen (Table 2.2 in Chapter 2) indicated that the

acetoclastic methanogens and propionate-degrading acetogenic bacteria were more sensitive to product inhibition than the butyrate-degrading acetogenic bacteria.

To determine whether acetate and propionate concentrations affected each other and their own degradation, plots of acetate concentration versus acetate and propionate degradations as well as propionate concentration versus acetate and propionate degradations were carried out.

(a) (b)



Figures 8.6 (a) and (b). Relationships between acetate concentration and acetate degradation as well as between acetate concentration and propionate degradation respectively

Figure 8.6 (a) and (b) show strong negative correlations (R^2 = -0.993 and -0.9216) existed between acetate degradation and its own concentration as well as between propionate degradation and acetate concentration respectively. These observations indicated that acetate at high concentration inhibited its own degradation as well as inhibited propionate degradation.

(a) (b)

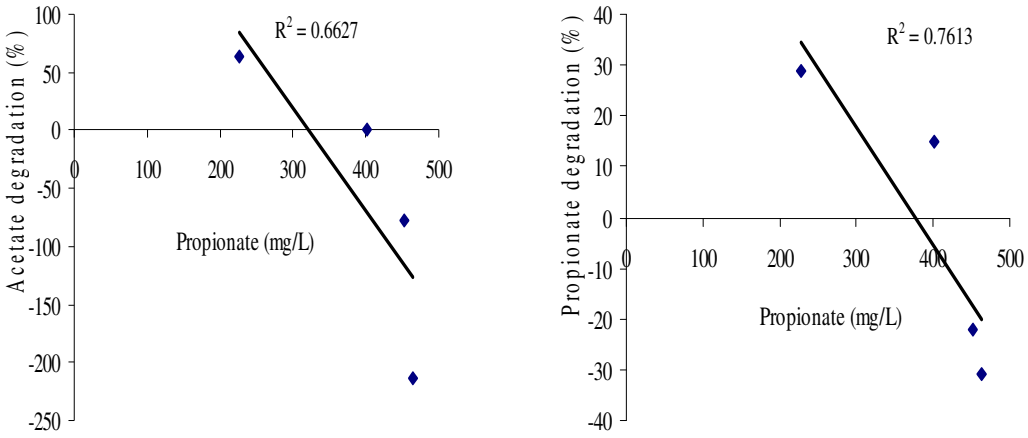


Figure 8.7 (a) and (b). Relationships between acetate concentration and propionate degradation as well as between propionate concentration and propionate degradation

Figure 8.7 (a) and (b) show moderate negative correlations (R^2 = -0.6627 and -0.7613) existed between acetate degradation and propionate concentration as well as between propionate degradation and its own concentration respectively. These observations indicated that inhibition of acetate and propionate degradation was not due solely to propionate concentration.

Relationships of ammonia with initial pH, VFA degradation and methane production

Table 8.3 gives the pH, ammonium-nitrogen and ammonia concentrations of the thermophilic piggery wastewater at the start and end of the digestion period while Figure 8.8 presents the ammonia and pH data graphically.

Table 8.3. pH, ammonium-nitrogen and free ammonia in the thermophilic piggery wastewater at start and end of the batch serum vial digestion experiment

Sample	Day	pH	$NH4^+$-N (mg/L)	Free NH_3 (mg/L)
pH 8.3	0	8.3	2104 (73)	916 (32)
	10	8.3 (0.1)	2088 (85)	853 (101)
pH 7.5	0	7.5	2097 (60)	228 (7)
	10	8.1 (0)	2291 (56)	686 (20)
pH 7	0	7	2052 (0)	76 (0)
	10	8.1 (0.1)	2125 (56)	643 (50)
pH 6.5	0	6.5	1976 (3)	24 (0)
	10	7.8 (0)	2171 (112)	425 (22)

Data are mean values of replicates (± standard deviation)

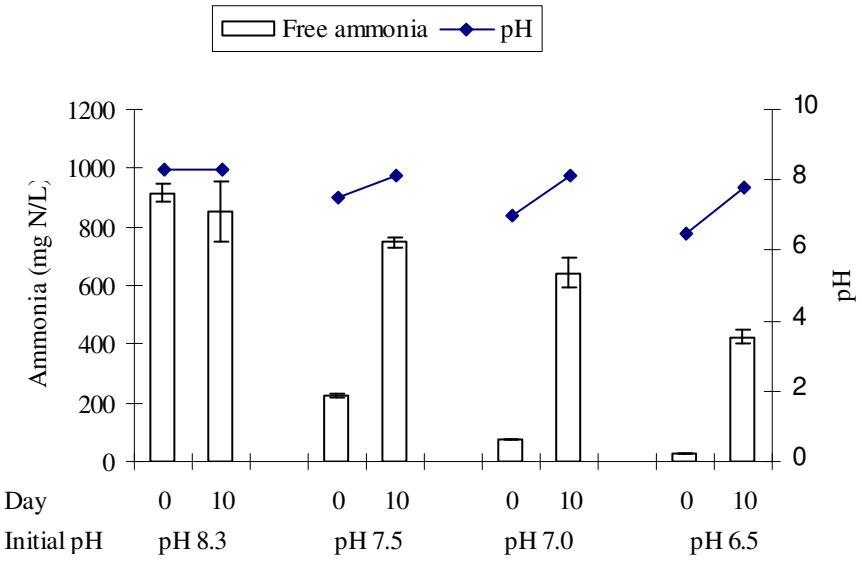


Figure 8.8. pH and free ammonia concentrations of the thermophilic piggery wastewaters (error bars indicate standard deviations)

The proportion of free dissolved ammonia concentration in the wastewater is influenced by pH and temperature (section 2.5.4 in Chapter 2). By reducing the initial piggery wastewater pH from pH 8.3 to 7.5, 7 and 6.5 at 55^{o}C, the high ammonia concentration was significantly reduced from 916 ± 32 to 24 ± 0 mg N/L at pH 6.5 (Figure 8.8). The low initial ammonia concentration at pH 7.5, 7 and 6.5 facilitated further degradation of proteinaceous organic materials as evidenced by the increased ammonia concentrations and pH.

To determine the relationships between initial pH, total VFA degradation, methane production and final free ammonia concentrations, the data of initial pH, total VFA degradation and methane production were plotted against their corresponding free ammonia data. Figure 8.9 shows a strong positive correlation (R^2 = 0.9401) existed between the final free ammonia and initial pH.

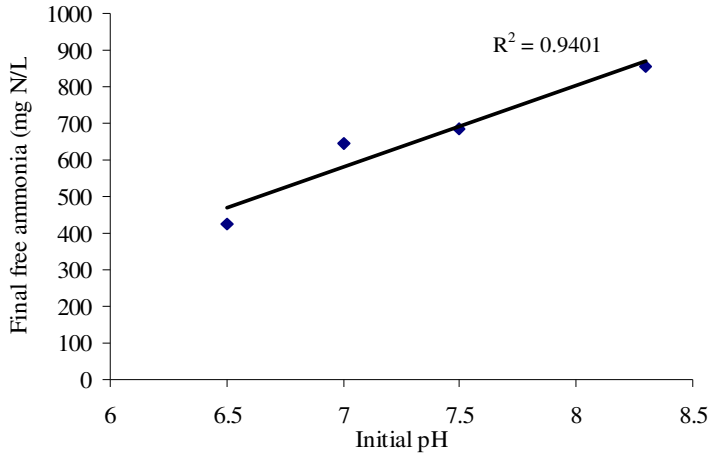


Figure 8.9. Relationship between final free ammonia and initial pH of the thermophilic piggery wastewater

Figure 8.10 shows both the TVFA-COD degradation and methane production exhibited strong negative correlation (R^2 = -0.9757 and -0.9686 respectively) with free ammonia concentration. The trends directly linked increasing free ammonia concentration to decreasing total VFA degradation and decreasing methane production.

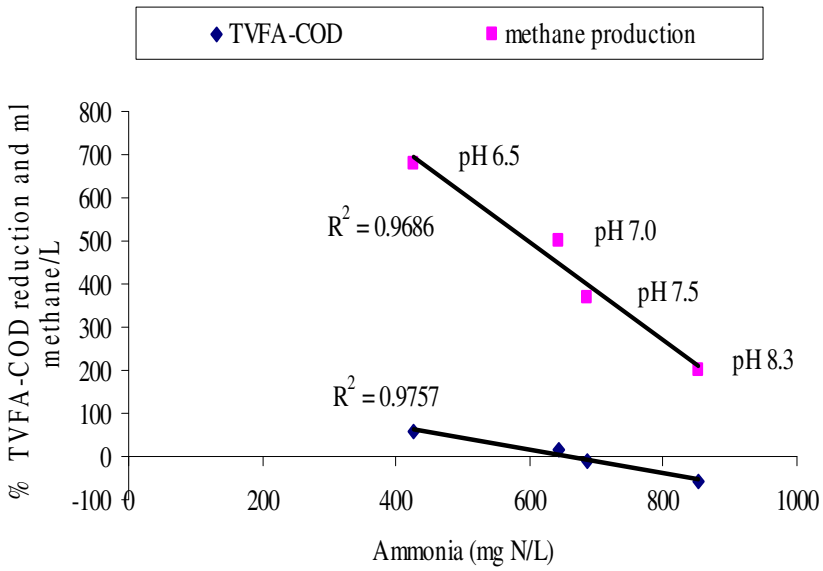


Figure 8.10. Relationships between free ammonia, methane production and total VFA-COD reduction in the thermophilic piggery wastewater at different pH

Similarly, plots of acetate and propionate degradation data versus their respectively free ammonia data in Figure 8.11 show both VFA degradation exhibited strong

negative correlation (R^2 = -0.905 and -0.8087 respectively) with free ammonia concentration. The trends directly linked increasing free ammonia concentration to decreasing amounts of acetate and propionate being degraded.

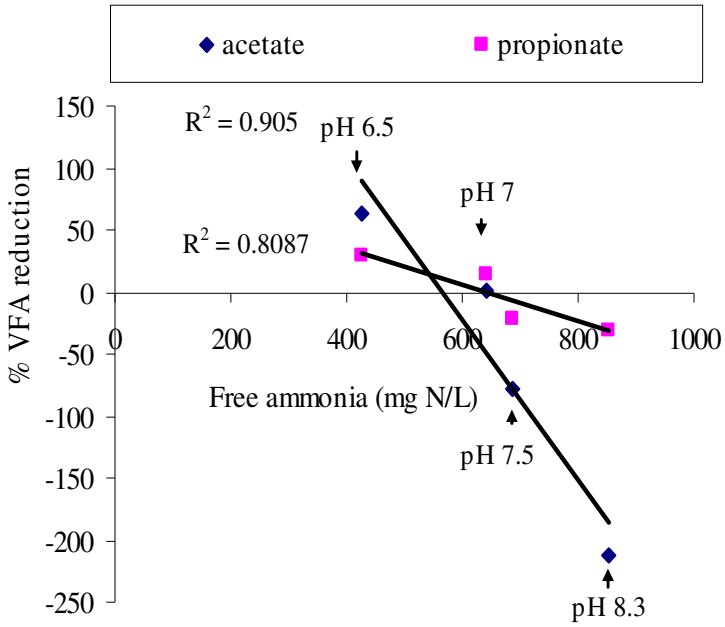


Figure 8.11. Relationships between free ammonia, acetate and propionate degradation in the thermophilic piggery wastewater at different pH

Degradations of butyrate (i- and n-) and valerate (i- and n-) were unaffected by the wastewater ammonia level as shown in Figure 8.5. The observation suggested that butyrate-degrading acetogenic bacteria which oxidise butyrate and higher carbon fatty acids were more resilient to product inhibition than the acetoclastic methanogens and syntrophic propionate-degrading acetogenic bacteria-hydrogenotrophic microorganisms.

Anaerobic microorganisms

T-RFLP profiling was carried out using eubacterial 16S primers 341F-FAM and 907R and restriction enzyme Alu I digestion. Means of 2-4 sample profiles were used to compare the bacterial group. Figure 8.12 shows the microbial diversity decreased with decreasing pH, with pH-unadjusted wastewater at pH 8.3 having the highest diversity as visualised by the number of fragments obtained. Increased proportions of microorganisms belonging to the predominant groups of VFA utilisers such as *Meiothermus silvanus, Thermotoga spp., Acinetobacter,*

Thermodesulfobacterium, and *Bacteroides* were observed whilst *Propionibacterium, Actinomycetes* and *Bifidobacterium* decreased in proportions.

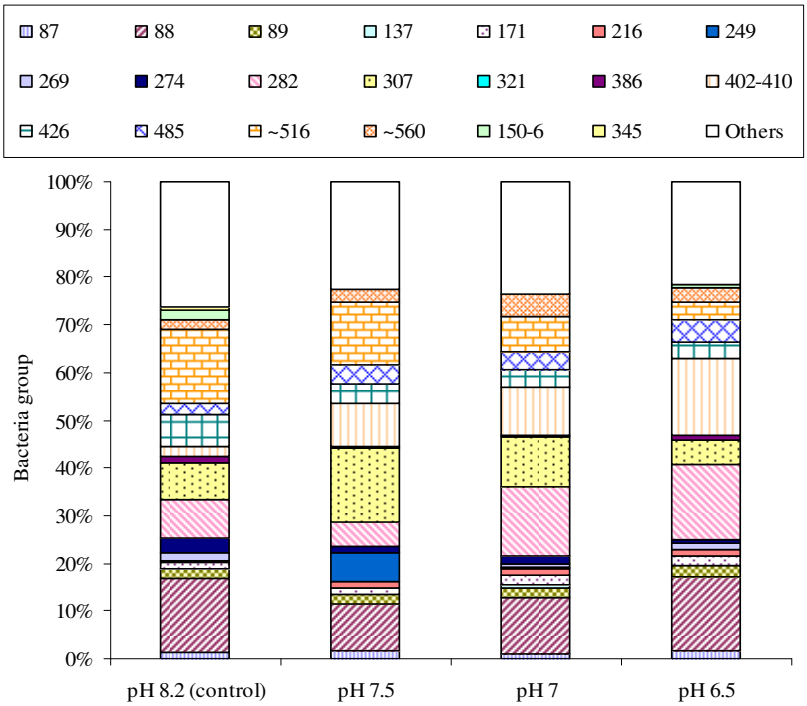


Figure 8.12. Approximate distribution of bacteria group in the thermophilic piggery wastewater at different pH at the end of the test (day 10)

With decreasing pH to 6.5, higher proportions of fermentative anaerobic groups such as *Bacteroides, Cytophaga spp, possibly Desulfovibrio* or *Desulfosporosinus* or *Bifidobacterium* were present. *Meiothermus ruber, Clostridium* and *Thiobacillus* were additional groups found at pH 7 and pH 7.5. Appendix 6 gives a list of the bacterial genera that belong to the designated fragment.

Methanosarcina thermophila, an acetoclastic methanogen which also consumes H_2/CO_2 predominated at high pH of 8.3 where elevated acetate concentration was observed. In contrast, hydrogen-utilising methanogens such as *Methanoculleus spp*. predominated at low pH of 6.5 that produced the highest acetate and propionate degradation. Figure 8.13 shows highest number of methanogens (8×10^5) was present at pH 6.5 which corresponded with the highest methane production (Figure 8.1.). It was also observed that the number of pathogenic *Clostridium perfringens* was lowest compared to the control at pH 8.3 (2×10^3 vs 2×10^4 at pH 8.3). It appeared that with increased methane production or methanogen population, there

was a concurrent reduction in the number of pathogenic *Clostridium perfringens* due possibly to the methanogens' superior competitive edge over *Clostridium* for common substrates.

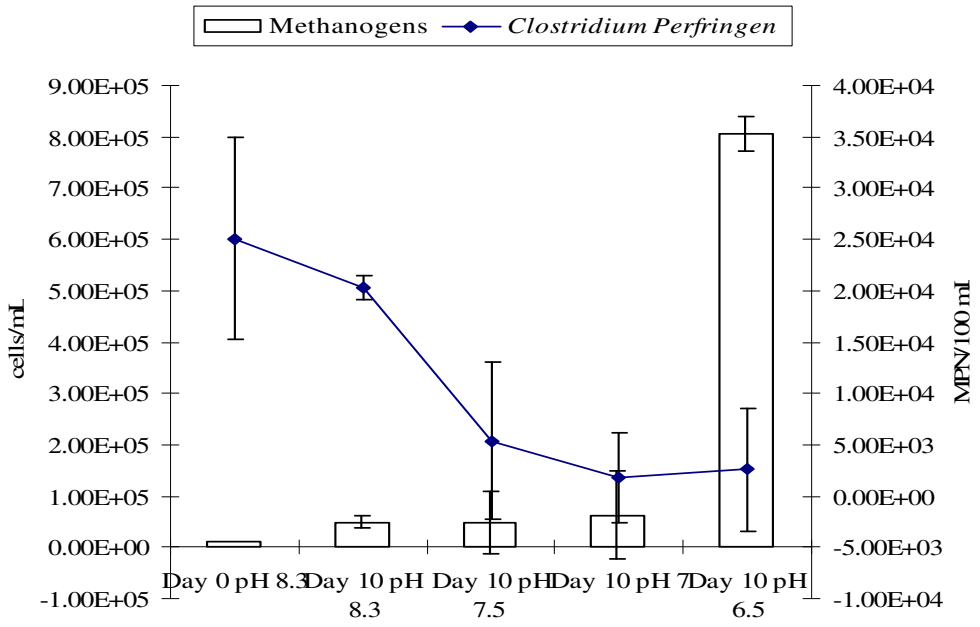


Figure 8.13. Approximate estimations of methanogens and *Clostridum perfringen* populations (error bars indicate standard errors)

8.3.2. Effect of biomass supplements on methane production from thermophilic batch anaerobic digestion of pH-unadjusted and pH-adjusted piggery wastewaters

8.3.2.1. Piggery biomass supplement

Table 8.4 gives the initial volatile solids and total COD concentrations of the pH-unadjusted (C1) and pH-reduced (C2) piggery wastewaters with and without piggery biomass supplements at the start of the experiment. Due to their organic nature, the inputs of 10% and 19% piggery biomass in the form of thick slurry to the piggery wastewater not surprisingly increased its total COD concentration. The higher the amount of biomass added as VS, the lower the ratio of total COD/VS becomes which implies that the substrate could become rate-limiting to the microbes at a certain threshold ratio. However, at the applied volumetric amount of 10% to 19% piggery biomass, their ratios of TCOD/VS were relatively high at 70-89% compared to the control. Based on these data, it is reasonable to assume that substrate

limitation would not pose a significant issue in this short-term batch digestion experiment.

Table 8.4. Wastewater volatile solids (VS) and total COD concentrations with and without piggery biomass (pb) supplements at the start of the experiment

Substrate (50 ml)	g-VS	g-TCOD	g-TCOD/g-VS
pH-unadjusted piggery wastewater (C1)	0.203	0.374	1.847
C1 + 10% piggery biomass	0.323	0.532	1.647
C1 + 19% piggery biomass	0.432	0.556	1.287
pH-reduced piggery wastewater (C2)	0.2	0.368	1.84
C2 + 10% piggery biomass	0.321	0.495	1.542
C2 + 19% piggery biomass	0.43	0.574	1.335

Data are mean values of replicates

Methane production

Figure 8.14 illustrates the methane yield from the pH-unadjusted (pH 8.1) and pH-reduced (pH 6.5) thermophilic piggery wastewaters with and without piggery biomass supplements.

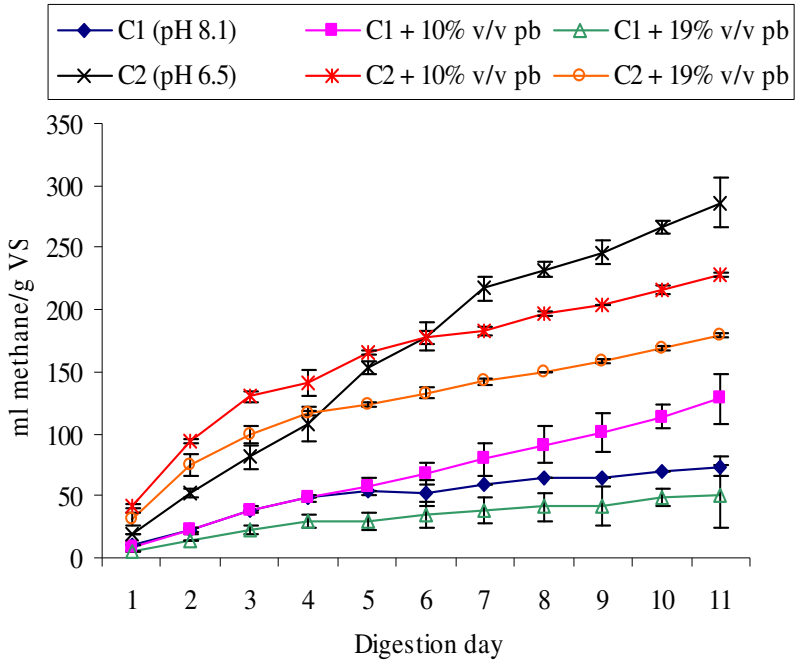


Figure 8.14. Effect of supplementing piggery biomass (pb) on methane production from pH-unadjusted (C1) and pH-reduced (C2) thermophilic piggery wastewaters (error bars indicate standard deviations)

Supplementing 10% v/v piggery biomass to the pH-unadjusted wastewater resulted in an increase in methane yield from 74 ± 7 ml/g VS to 128 ± 20 ml/g VS at day 11. However, at higher amount of 19% v/v piggery biomass, methane yield was reduced to 50 ± 25 ml/g VS which indicated that the biomass was under stress probably from inhibition by its high initial free ammonia (Table 8.7).

Reducing the wastewater pH alone from pH 8.1 to pH 6.5 greatly enhanced the methane yield by four-fold. Supplementing 10% and 19% piggery biomass to the pH-reduced piggery wastewater produced only small increases in methane yields of 141 ± 11 ml/g VS and 117 ± 2 ml/g VS respectively compared to 108 ± 14 ml/g VS of the control on day 3. However, methane production began to slow down thereafter as shown by the reduced methane yields compared to the pH-reduced control. By the end of the experiment (day 11), methane yields of the pH-reduced wastewater supplemented with piggery biomass had lagged behind the control pH-reduced wastewater (228 ± 2 ml/g VS with 10% biomass and 179 ± 2 ml/g VS with 19% biomass versus 286 ± 20 ml/g VS control).

For both the pH-unadjusted and pH-reduced piggery wastewaters, supplementing twice as much piggery biomass (19%) did not result in greater methane production enhancement over 10% biomass supplement. Instead, greater reduction of methane production with time was observed compared to the control.

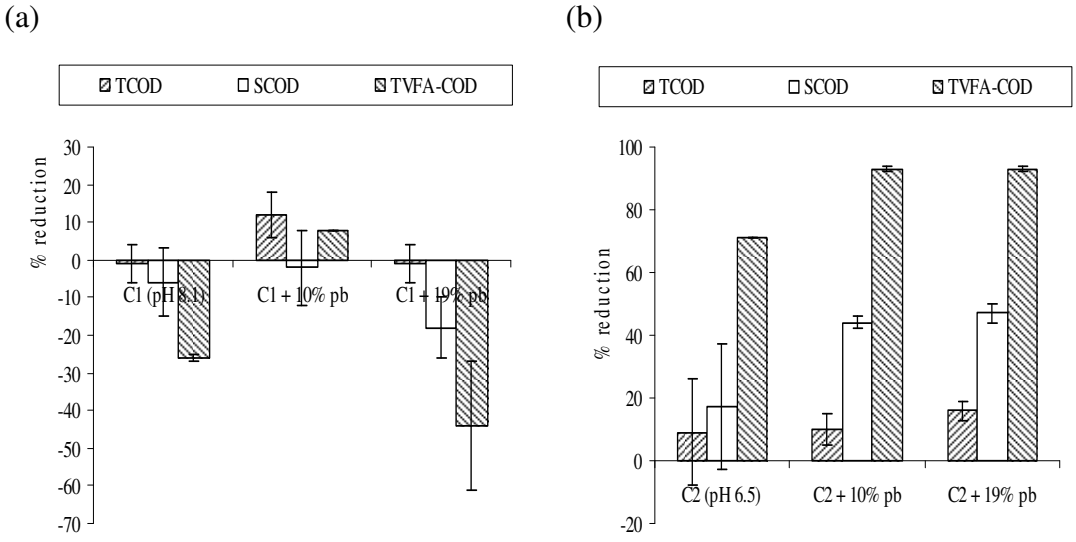
Organics reduction (TCOD, SCOD and TVFA-SCOD)

Table 8.5 gives the COD (total and soluble) and total VFA-COD concentrations of the pH-unadjusted (pH 8.1) and pH-reduced (pH 6.5) thermophilic wastewaters at the start and end of the test period. The percentage reductions of wastewater total COD, soluble COD and total VFA-SCOD were graphically presented in Figures 8.15 (a) and (b) for the pH-unadjusted and pH-reduced thermophilic wastewaters respectively. Negative percentage reductions indicate the accumulation of organics.

Table 8.5. Chemical oxygen demand (total and soluble) and total VFA concentrations of the pH-unadjusted (C1) and pH-adjusted (C2) piggery wastewaters

Sample	Day	TCOD (mg/L)	SCOD (mg/L)	TVFA (mg COD/L)
C1 (pH 8.1)	0	7480 (421)	3222 (185)	2132 (167)
C1 (pH 8.1)	11	7568 (361)	3411 (274)	2683 (31)
C1 + 10% pb	0	10631 (729)	3259 (132)	1977 (167)
C1 + 10% pb	11	9369 (669)	3327 (336)	1829 (9)
C1 + 19% pb	0	11123 (557)	3538 (158)	2046 (49)
C1 + 19% pb	11	11262 (569)	4173 (269)	2948 (355)
C2 (pH 6.5)	0	7359 (631)	3352 (263)	2118 (16)
C2 (pH 6.5)	11	6667 (1258)	2779 (656)	616 (9)
C2 + 10% pb	0	9909 (811)	3632 (26)	1883 (89)
C2 + 10% pb	11	8919 (540)	2017 (56)	135 (19)
C2 + 19% pb	0	11487 (303)	3482 (79)	1908 (3)
C2 + 19% pb	11	9676 (388)	1859 (91)	135 (19)

Data are mean values of replicates (± standard deviation) pb (piggery biomass)



Figures 8.15 (a) and (b). Effects of piggery biomass (pb) supplements on COD (total and soluble) and total TVFA reductions in pH-unadjusted (C1) and pH-reduced (C2) thermophilic piggery wastewaters respectively (error bars indicate standard deviations)

As shown in Figure 8.15 (a), supplementing 10% v/v piggery biomass to the pH-unadjusted piggery wastewater resulted in some 10% increase in TCOD and TVFA-COD reductions which corresponded with the enhanced methane yield (Figure 8.14). However, in the case of the 19% piggery biomass-spplemented wastewater, methane production and reductions of COD and TVFA were observed to drop.

Figure 8.15 (b) shows reductions of wastewater COD and total VFA-COD were substantially higher in the pH-reduced control (C2). Supplementing 10% and 19% v/v piggery biomass to the pH-reduced wastewater further increased the SCOD and TVFA-COD reductions by 27-30% and 22% respectively, with the increased COD reductions comparable between 10% and 19% v/v piggery biomass supplements. The enhanced COD reductions however, were inconsistent with their reduced methane yields relative to the control at the end of the digestion period (Figure 8.14). The negative correlation (R^2 = -0.7908) between TVFA-COD reduction and methane yield data suggested that the observed inconsistency could be caused by three factors: 1) some volatile fatty acids being consumed by non-methanogenic anaerobic microorganisms; 2) microbial consumption of the easily degradable methanogenic substrates had resulted in a growth-limiting unfavourable environment that contained non-biodegradable organic substrates; or 3) some biogas had leaked through the rubber stoppers which were poked daily for biogas measurement.

Volatile fatty acids degradation

Table 8.6 gives the VFA components present in the soluble organic fraction at the start and end of the test period while Figures 8.16 (a) and (b) show the percentage VFA degradation in the pH-unadjusted and pH-reduced thermophilic wastewaters respectively. Negative percentage degradation indicates VFA accumulation.

Supplementing 10% piggery biomass to the wastewater greatly improved acetate degradation but not propionate although the final acetate level was still elevated (Figure 8.16 (a)). The improvement in acetate degradation correlated positively (R^2 = 0.9979) with the increased methane yield. However, there were no noticeable improvements in acetate and proiopnate degradation with the higher supplement of 19% piggery biomass.

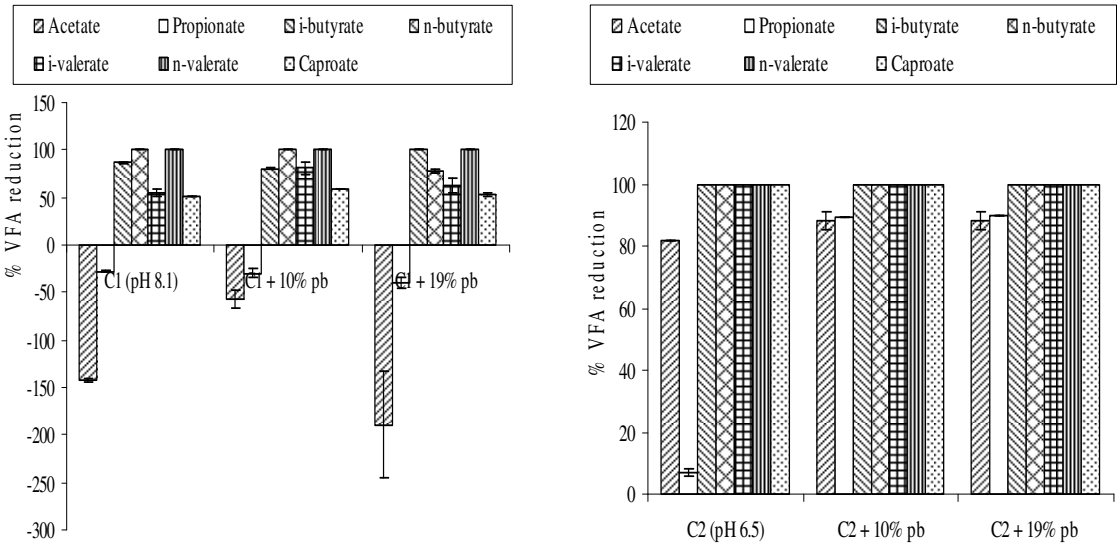
Table 8.6. Volatile fatty acid concentrations in the thermophilic piggery wastewater at the start and end of the test period

Sample	Day	Acetate (mg/L)	Propionate (mg/L)	i-butyrate (mg/L)	n-butyrate (mg/L)	i-valerate (mg/L)	n-valerate (mg/L)	Caproate (mg/L)
C1 (pH 8.1)	0	693 (73)	355 (32)	95 (4)	77 (8)	167 (10)	71 (4)	29 (4)
C1 (pH 8.1)	11	1681 (11)	454 (5)	12 (1)	0	75 (4)	0	14 (0
C1 + 10% pb	0	599 (100)	360 (33)	87 (5)	68 (5)	155 (4)	63 (2)	32 (1)
C1 + 10% pb	11	940 (54)	467 (18)	18 (1)	0	30 (11)	0	13 (0)
C1 + 19% pb	0	620 (8)	406 (13)	79 (3)	65 (2)	154 (3)	63 (2)	32 (1)
C1 + 19% pb	11	1795 (346)	569 24)	0	14 (1)	58 (11)	0	15 (1)
C2 (pH 6.5)	0	690 (13)	343 (3)	91 (1)	78 (1)	167 (1)	73 (1)	32 (0)
C2 (pH 6.5)	11	124 (0)	320 (0)	0	0	0	0	0
C2 + 10% pb	0	584 (47)	341 (18)	79 (2)	65 (4)	147 (1)	61 (4)	29 (0)
C2 + 10% pb	11	71 (16)	39 (1)	0	0	0	0	0
C2 + 19% pb	0	586 (3)	374 (4)	71 (1)	60 (0)	144 (2)	59 (0)	31 (1)
C2 + 19% pb	11	71 (16)	39 (1)	0	0	0	0	0

C1 (pH-unadjusted) C2 (pH-reduced) pb (piggery biomass)

Data are mean values of replicates (± standard deviation)

(a) (b)



Figures 8.16 (a) and (b). Effect of piggery biomass (pb) supplements on VFA degradation in pH-unadjusted (C1) and pH-reduced (C2) thernophilic piggery wastewaters respectively (error bars indicate standard deviations)

Figure 8.16 (b) shows pH reduction alone of the piggery wastewater from pH 8.1 to pH 6.5 greatly enhanced the reductions of acetate, propionate, i-butyrate, i-valerate and caproate concentrations. Supplementing 10% and 19% piggery biomass substantially enhanced propionate degradation and resulted in significantly higher

TVFA-COD reductions over the control (Figure 8.15 (b)). However, the negative correlations between TVFA-COD reduction and methane yield data as well as between acetate reduction and methane yield data (both R^2 = -0.7908) implied that the improvements in acetate and propionate degradation in the pH-reduced wastewater supplemented with piggery biomass (10% and 19%) could possibly be due to a combination of increased microbial activity from non-methanogenic bacteria such as sulphate-reducing bacteria (SRB) and easily degradable substrate limitation.

Ammonia, pH and relationships of ammonia with VFA degradation and methane yield

Table 8.7 gives the pH, ionised ammonium-nitrogen and unionised free ammonia concentrations of the pH-unadjusted (C1) and pH-reduced piggery wastewaters (C2) with and without piggery biomass supplements at the start and end of the digestion period. At the end of the digestion period, both controls (C1 and C2) showed significant increase in pH and free ammonia concentration as a result of microbial degradation of the wastewater nitrogenous proteins and urea.

Table 8.7. pH, ammonium-nitrogen and dissolved free ammonia concentrations in pH-unadjusted (C1) and pH-reduced (C2) digested piggery wastewaters without and with piggery biomass (pb) supplement

Sample	Day	pH	NH_4^+-N (mg/L)	Free ammonia (mg/L)
C1 (pH 8.1)	0	8.1	2260 (22)	739 (7)
C1 (pH 8.1)	11	8.3 (0)	2218 (70)	966 (31)
C1 + 10% pb	0	8.3	2064 (32)	896 (14)
C1 + 10% pb	11	8.4 (0)	2369 (95)	1167 (47)
C1 + 19% pb	0	8.4	2134 (19)	1051 (10)
C1 + 19% pb	11	8.4 (0)	2241 (10)	1103 (5)
C2 (pH 6.5)	0	6.5	2174 (7)	26 (0)
C2 (pH 6.5)	11	7.9 (0.1)	2272 (111)	533 (26)
C2 + 10% pb	0	6.5	2169 (15)	26 (0)
C2 + 10% pb	11	7.8 (0)	2313 (92)	453 (18)
C2 + 19% pb	0	6.6	1978 (27)	30 (0)
C2 + 19% pb	11	7.9 (0.1)	2361 (63)	554 (15)

Data are mean values of replicates (± standard deviation)

Addition of 10% and 19% piggery biomass to the pH-unadjusted wastewater was observed to raise the initial pH by 0.2 and 0.3 respectively as well as increased the

free ammonia concentrations. The higher initial free ammonia concentration of the 19% biomass supplemented wastewater compared to the control and 10% biomass supplemented wastewater probably contributed to the methane yield inhibition observed (Figure 8.14). Plots of TVFA-COD reduction and methane yield against initial free ammonia data (Figure 8.17) show that while there was no correlation between either total VFA-COD reduction (R^2 = 0.1138) or methane yield (R^2 = 0.0881) and initial free ammonia concentration, it appears there existed a free ammonia threshold of between 700 and 900 mg N/L in which VFA degradation and methane yield were observed to be adversely affected. It is noted that free ammonia concentrations of the pH-unadjusted wastewater with and without biomass supplements were all high.

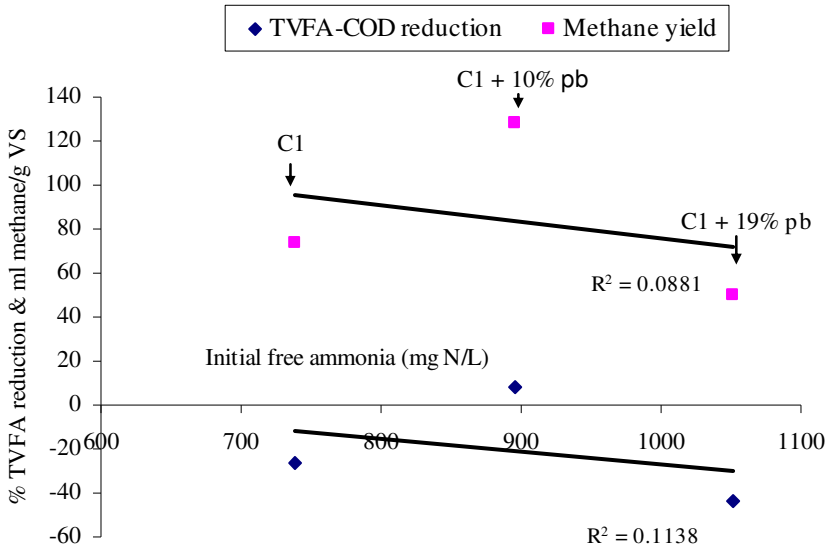


Figure 8.17. Relationships between total VFA-COD reduction, methane yield and initial free ammonia concentration in the pH-unadjusted (C1) piggery wastewater

Reducing the piggery wastewater pH from its initial pH of 8.1 to pH 6.5 alone significantly lowered the high free ammonia concentration by 96% and facilitated further degradation of the organic materials. The latter was reflected by the increase in pH and ammonia-nitrogen (Table 8.7) as well as four-fold increase in methane yield (Figure 8.14) at the end of the digestion period. Plots of total VFA-COD reduction and methane yield against final free ammonia data of the pH-reduced wastewaters with and without piggery biomass supplement (Figure 8.18) show there was no correlation existing between either total VFA-COD reduction (R^2 = 0.1021)

or methane yield (R^2 = 0.0223) and final free ammonia concentration. This implied that the observed inconsistencies of higher TVFA reductions and lower methane yields from the 10% and 19% biomass-supplemented pH-reduced wastewaters were not due to ammonia inhibition but possibly other inhibitor such as hydrogen sulphide which was not measured in this study.

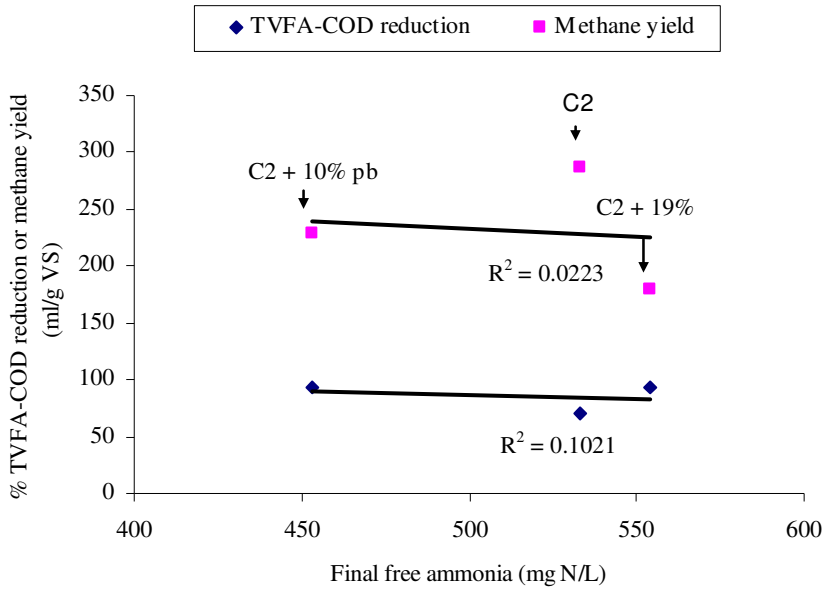


Figure 8.18. Relationships between total VFA-COD reduction, methane yield and final free ammonia concentration in the pH-reduced (C2) piggery wastewater

8.3.2.2. DiCOM biomass supplement

Table 8.8 gives the initial volatile solids and total COD concentrations of the pH-unadjusted (C1) and pH-reduced (C2) piggery wastewaters with and without DiCOM biomass supplements at the start of the experiment. Due to their organic nature, the inputs of 10% and 19% DiCOM biomass in the form of thick slurry to the piggery wastewater not surprisingly increased its total COD concentration significantly. Similar to the piggery biomass, the higher the amount of biomass added as VS, the smaller the ratio of total COD/VS becomes which implies that the substrate could become rate-limiting to the microbes at a certain threshold ratio. However, at the applied volumetric amount of 10% to 19% DiCOM biomass, their ratios of TCOD/VS were relatively high at 71-82% compared to the control. Based on these data, it is reasonable to assume that substrate limitation would not pose a significant issue in this short-term batch digestion experiment.

Table 8.8. Wastewater volatile solids (VS) and total COD concentrations with and without DiCOM biomass (db) supplements at the start of the experiment

Substrate (50 ml)	g-VS	g-TCOD	g-TCOD/g-VS
pH-unadjusted piggery wastewater (C1)	0.203	0.374	1.847
C1 + 10% DiCOM biomass	0.493	0.714	1.448
C1 + 19% DiCOM biomass	0.755	0.996	1.319
pH-reduced piggery wastewater (C2)	0.2	0.368	1.84
C2 + 10% DiCOM biomass	0.491	0.744	1.515
C2 + 19% DiCOM biomass	0.753	1.015	1.348

Data are mean values of replicates

Methane production

Figure 8.19 illustrates the methane yield from the pH-unadjusted (pH 8.1) and pH-reduced (pH 6.5) thermophilic piggery wastewaters with and without piggery biomass supplements.

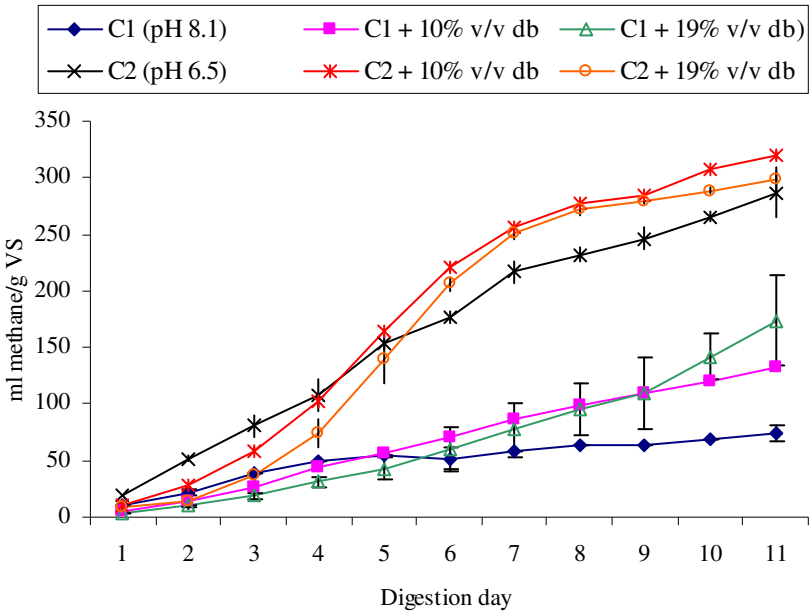


Figure 8.19. Effect of supplementing DiCOM biomass (db) on methane production from pH-unadjusted (C1) and pH-reduced (C2) thermophilic piggery wastewaters (error bars indicate standard deviations)

Supplementing 10% and 19% DiCOM biomass to the pH-unadjusted wastewater (C1) resulted in an increase in methane yield from 74 ± 7 ml/g VS to 132 ± 3 ml/g VS and 174 ± 40 ml/g VS at day 11. The methane yields of 10% and 19% biomass supplements were however comparable. An initial lag period of about 3 days for

10% biomass and about 5 days for 19% biomass was apparent in the methane yields before they exceeded the control. The lag period reflected the duration it took the DiCOM microorganisms to get acclimatised to the new environment.

Reducing the wastewater pH alone from pH 8.1 to pH 6.5 greatly enhanced the methane yield by four-fold. Supplementing 10% DiCOM biomass to the pH-reduced piggery wastewater produced only small increase of 12% in methane yield (320 ± 3 ml/g VS) over the control (286 ± 20 ml/g VS) although it was significantly greater than the methane yield of 19% DiCOM biomass (299 ± 11 ml/g VS) on day 11. An initial lag period of about 3 days with 10% biomass and about 5 days with 19% biomass was observed in the methane yields before they exceeded the control.

For both the pH-unadjusted and pH-reduced piggery wastewaters, increasing the amount of DiCOM biomass supplement from 10% to 19% did not result in greater enhancement of methane production.

Organic reduction (TCOD, SCOD and TVFA-SCOD)

Table 8.9 gives the COD (total and soluble) and total VFA-COD concentrations of the pH-unadjusted (pH 8.1) and pH-reduced (pH 6.5) thermophilic wastewaters at the start and end of the test period. The percentage reductions of wastewater total COD, soluble COD and total VFA-SCOD were graphically presented in Figures 8.20 (a) and (b) for the pH-unadjusted and pH-reduced thermophilic wastewaters respectively. Negative percentage reductions indicate the accumulation of organics.

As shown in Figure 8.20 (a), supplementing 10% and 19% DiCOM biomass to the pH-unadjusted piggery wastewater resulted in significant increase in TVFA reductions over the control. These were reflected in the observed enhancement in methane yield (Figure 8.19). Correlation between the amount of TVFA-COD degraded and methane production was moderately positive (R^2 = 0.6559) which implied that part of the organic substrates degraded were not available for conversion to methane, possibly due to substrate consumption by non-methanogenic bacteria. The inconsistency in SCOD reduction with TVFA reduction and methane yield of the 10% biomass-supplement wastewater (C1) was likely due to measurement error being incurred in the COD analysis.

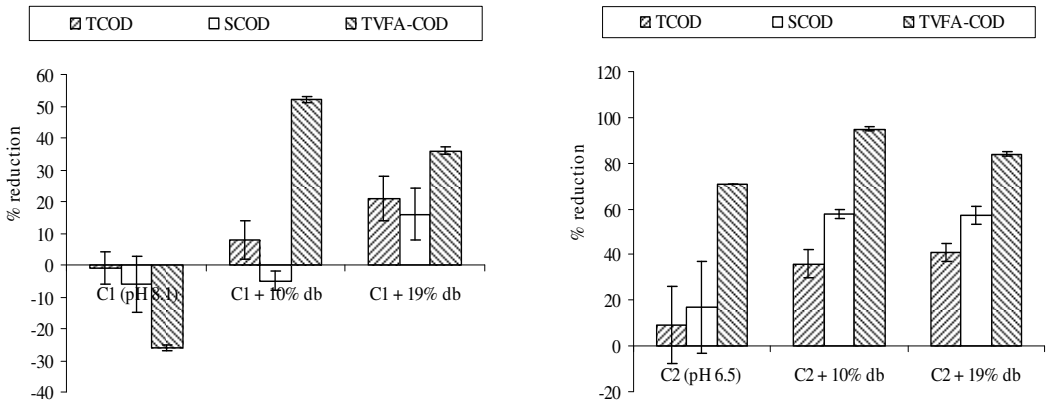
Table 8.9. Chemical oxygen demand (total and soluble) and total volatile fatty acids at start and end of the batch serum vial digestion experiment with DiCOM biomass addition

Sample	Day	TCOD (mg/L)	SCOD (mg/L)	TVFA (mg COD/L)
C1 (pH 8.1)	0	7480 (421)	3222 (185)	2132 (167)
C1 (pH 8.1)	11	7568 (361)	3411 (274)	2683 (31)
C1 + 10% db	0	14280 (365)	6239 (26)	5365 (171)
C1 + 10% db	11	13153 (857)	6555 (215)	2602 (45)
C1 + 19% db	0	19914 (748)	8343 (474)	8000 (397)
C1 + 19% db	11	15676 (1396)	6989 (662)	5153 (1000)
C2 (pH 6.5)	0	7359 (631)	3352 (263)	2118 (16)
C2 (pH 6.5)	11	6667 (1258)	2779 (656)	616 (9)
C2 + 10% db	0	14887 (256)	6146 (527)	4880 (215)
C2 + 10% db	11	9550 (953)	2602 (129)	266 (29)
C2 + 19% db	0	20303 (607)	8529 (36)	6736 (504)
C2 + 19% db	11	11892 (900)	3643 (331)	1084 (101)

C1 (pH-unadjusted) C2 (pH-reduced) db (DiCOM biomass)

Data are mean values of replicates (± standard deviation)

(a) (b)



Figures 8.20 (a) and (b). Effect of DiCOM biomass (db) supplements on COD (total and soluble) and total TVFA reductions in pH-unadjusted (C1) and pH-reduced (C2) thermophilic piggery wastewaters respectively (error bars indicate standard deviations)

Figure 8.20 (b) shows reduction of wastewater pH from 8.1 to 6.5 alone greatly enhanced total VFA-COD degradation. Supplementing 10% and 19% v/v DiCOM biomass to the pH-reduced wastewater further increased the TVFA-COD reductions by 24% and 13% respectively compared to the control. The enhanced COD reductions were consistent with their enhanced methane yields at the end of the

digestion period (Figure 8.19). Correlation between TVFA-COD reduction and methane yield data was strongly positive ($R^2 = 0.9668$).

Volatile fatty acid degradation

Table 8.10 gives the VFA components present in the soluble organic fraction at the start and end of the test period while Figures 8.21 (a) and (b) show the percentage VFA degradation in the pH-unadjusted and pH-reduced thermophilic wastewaters respectively. Negative percentage degradation (reduction) indicates VFA accumulation.

Table 8.10. Volatile fatty acid concentrations in the thermophilic piggery wastewater at the start and end of the test period

Sample	Day	Acetate (mg/L)	Propionate (mg/L)	i-butyrate (mg/L)	n-butyrate (mg/L)	i-valerate (mg/L)	n-valerate (mg/L)	Caproate (mg/L)
C1 (pH 8.1)	0	693 (73)	355 (32)	95 (4)	77 (8)	167 (10)	71 (4)	29 (4)
C1 (pH 8.1)	11	1681 (11)	454 (5)	12 (1)	0	75 (4)	0	14 (0)
C1 + 10% db	0	1159 (11)	498 (10)	233 (16)	962 (37)	272 (16)	103 (4)	201 (14)
C1 + 10% db	11	1618 (33)	360 (1)	0	15 (1)	124 (4)	14 (1)	13 (1)
C1 + 19% db	0	1574 (61)	610 (30)	336 (14)	1706 (87)	349 (18)	140 (5)	317 (35)
C1 + 19% db	11	2381 (100)	915 (46)	80 (0)	105 (0)	363 (30)	46 (0)	29 (1)
C2 (pH 6.5)	0	690 (13)	343 (3)	91 (1)	78 (1)	167 (1)	73 (1)	32 (0)
C2 (pH 6.5)	11	124 (0)	320 (4)	0	0	0	0	0
C2 + 10% db	0	1085 (33)	462 (13)	201 (6)	856 (57)	252 (8)	96 (4)	180 (10)
C2 + 10% db	11	155 (10)	67 (26)	0	0	0	0	0
C2 + 19% db	0	1280 (64)	517 (45)	302 (10)	1414 (104)	310 (20)	119 (6)	296 (17)
C2 + 19% db	11	275 (91)	490 (0)	0	0	0	0	22 (0)

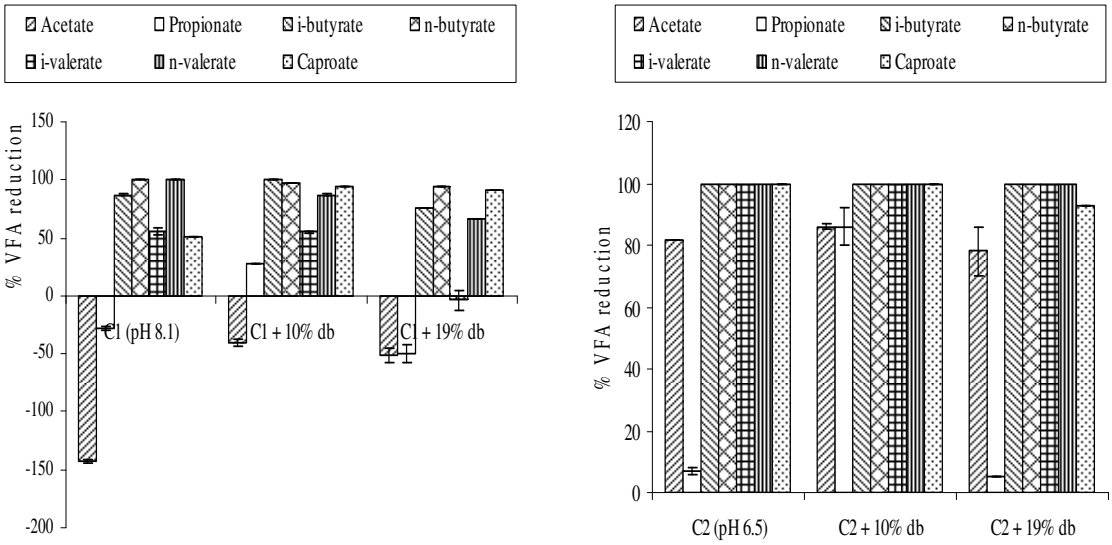
C1 (pH-unadjusted) C2 (pH-reduced) db (DiCOM biomass)

Data are mean values of replicates (± standard deviation)

In contrast to the piggery biomass, addition of DiCOM biomass was observed to greatly elevate the initial C2 to C6 volatile fatty acid concentrations of the piggery wastewater, with 19% biomass supplement elevating the VFA concentrations more than the 10% biomass. However, significantly improvements in acetate degradation were observed in the pH-unadjusted wastewater (C1) although their final acetate concentrations were still elevated compared to their initial concentrations (Figure 8.21 (a)). Positive correlation ($R^2 = 0.7454$) was observed between the amount of acetate degraded and the methane yield. Propionate, i-butyrate and caproate

degradations were also greatly improved in the pH-unadjusted wastewater with 10% DiCOM biomass supplement while no improvements in propionate and i-valerate degradation over the control were observed in the wastewater with 19% DiCOM biomass supplement.

(a) (b)



Figures 8.21 (a) and (b). Effect of DICOM biomass (db) supplements on VFA degradation in pH-unadjusted (C1) and pH-reduced (C2) thermophilic piggery wastewaters (error bars indicate standard deviations)

Figure 8.21 (b) shows pH reduction alone of the piggery wastewater from pH 8.1 to pH 6.5 greatly enhanced acetate, propionate, i-butyrate, i-valerate and caproate degradation. Supplementing 10% DICOM biomass vastly enhanced propionate degradation and resulted in significantly higher TVFA-COD reduction over the control (C2) and the 19% DiCOM biomass-supplemented wastewater (Figure 8.20 (b)). No significant improvement in propionate degradation was observed with higher supplement of DiCOM biomass (19%) compared to the control (C2).

Ammonia, pH and relationships of ammonia with VFA degradation and methane yield

Table 8.11 gives the pH, ionised ammonium-nitrogen and unionised free ammonia concentrations of the pH-unadjusted and pH-reduced piggery wastewaters with and without DiCOM biomass supplements at the start and end of the digestion period. At the end of the digestion period, both controls (C1 and C2) showed significant

increase in pH and free ammonia concentrations as a result of microbial degradation of the wastewater nitrogenous proteins and urea.

Table 8.11. pH, ammonium-nitrogen and dissolved free ammonia concentrations in pH-unadjusted (C1) and pH-reduced (C2) digested piggery wastewaters without and with DiCOM biomass (db) supplement

Sample	Day	pH	NH_4^+-N (mg/L)	Free ammonia (mg/L)
C1 (pH 8.1)	0	8.1	2260 (22)	739 (7)
C1 (pH 8.1)	11	8.3 (0)	2218 (70)	966 (31)
C1 + 10% db	0	8.2	2248 (10)	853 (4)
C1 + 10% db	11	8.2 (0)	2475 (46)	940 (17)
C1 + 19% db	0	8.2	2285 (7)	868 (7)
C1 + 19% db	11	8.2 (0.1)	2513 (41)	954 (16)
C2 (pH 6.5)	0	6.5	2174 (7)	26 (0)
C2 (pH 6.5)	11	7.9 (0.1)	2272 (111)	533 (26)
C2 + 10% db	0	6.5	2294 (36)	28 (0)
C2 + 10% db	11	7.9 (0)	2436 (44)	572 (10)
C2 + 19% db	0	6.5	1982 (7)	24 (0)
C2 + 19% db	11	7.8 (0)	2590 (48)	508 (10)

Data are mean values of replicates (± standard deviation)

Additions of 10% and 19% DiCOM biomass to the pH-unadjusted wastewater (C1) were observed to raise the initial pH by 0.1 as well as increased the free ammonia concentrations. Figure 8.22 shows the relationships between initial free ammonia concentration and TVFA-COD reduction as well as methane yield of the biomass-supplemented wastewater without pH reduction. The observed strong positive correlation between total VFA-COD reduction and free ammonia concentration (R^2 = 0.9116) as well as between methane yield and free ammonia concentration (R^2 = 0.8981) indicated that TVFA reduction and methane yield of the biomass-supplemented wastewaters were not affected by the high initial free ammonia concentrations.

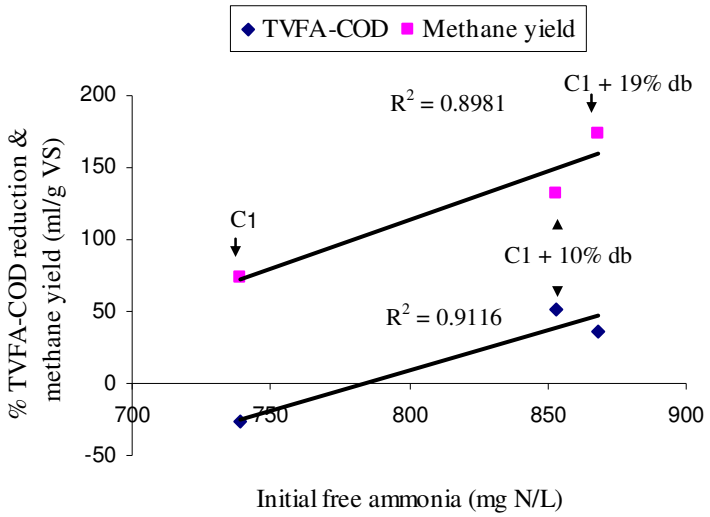


Figure 8.22. Relationships between total VFA-COD reduction, methane yield and initial free ammonia concentration in the pH-unadjusted (C1) piggery wastewater

Reducing the piggery wastewater pH from its initial pH of 8.1 to pH 6.5 alone significantly lowered the high free ammonia concentration by 96% and facilitated further degradation of the organic materials. The latter was reflected by the increase in pH and ammonia-nitrogen (Table 8.11) as well as four-fold increase in methane yield (Figure 8.19) at the end of the digestion period. Plots of total VFA-COD reduction and methane yield against final free ammonia data (Figure 8.23) show weak positive correlation existed between either total VFA-COD reduction (R^2 = 0.3199) or methane yield (R^2 = 0.4989) and final free ammonia concentration. This indicated that free ammonia at these concentrations did not adversely affect TVFA reductions and methane yields of the 10% and 19% biomass-supplemented pH-reduced wastewaters (C2).

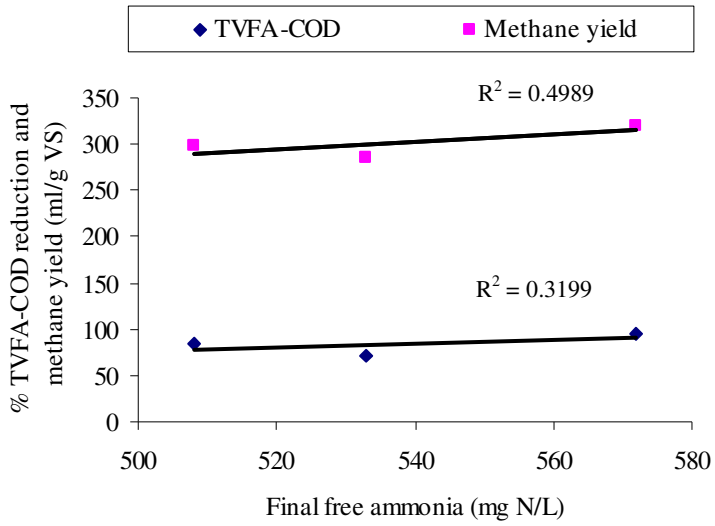


Figure 8.23. Relationships between total VFA-COD reduction, methane yield and initial final ammonia concentration in the pH-reduced (C2) piggery wastewater

8.3.2.3. Comparison of the digestion efficiency between piggery biomass and DiCOM biomass

Table 8.12 compares the initial volatile solids and total COD concentrations of the pH-unadjusted (C1) and pH-reduced (C2) piggery wastewaters with and without piggery biomass and DiCOM biomass supplements at the start of the experiment.

Table 8.12. Wastewater volatile solids (VS) and total COD concentrations with and without piggery biomass (pb) and DiCOM biomass (db) supplements at the start of the experiment

Substrate (50 ml)	g-VS	g-TCOD	g-TCOD/g-VS
pH-unadjusted piggery wastewater (C1)	0.203	0.374	1.847
C1 + 10% piggery biomass	0.323	0.532	1.647
C1 + 10% DiCOM biomass	0.493	0.714	1.448
C1 + 19% piggery biomass	0.432	0.556	1.287
C1 + 19% DiCOM biomass	0.755	0.996	1.319
pH-reduced piggery wastewater (C2)	0.2	0.368	1.84
C2 + 10% piggery biomass	0.321	0.495	1.542
C2 + 10% DiCOM biomass	0.491	0.744	1.515
C2 + 19% piggery biomass	0.43	0.574	1.335
C2 + 19% DiCOM biomass	0.753	1.015	1.348

Data are mean values of replicates

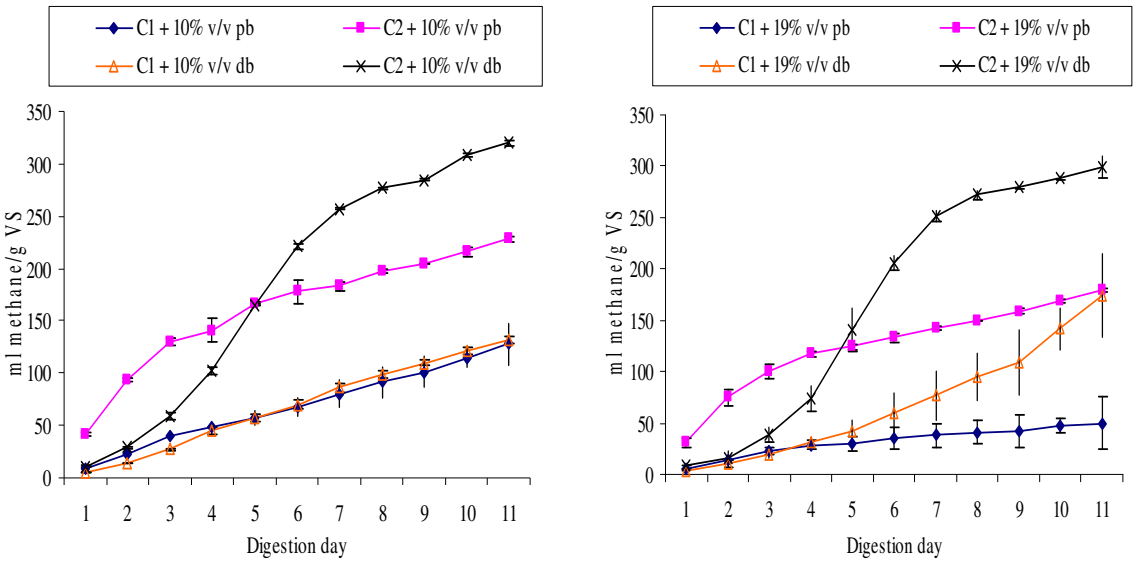
It was observed that while the addition of DiCOM biomass raised the organic content of the piggery wastewater more than the piggery biomass at similar volumes, their ratios of TCOD:VS were relatively similar. Based on these observations, it was

reasonable to conclude that the digestion performance comparison between the two types of biomass was not being compromised.

Methane production

Figures 8.24 (a) and (b) compare the methane yields of the piggery biomass- and DiCOM biomass-supplemented wastewaters without and with pH reduction respectively.

(a) (b)



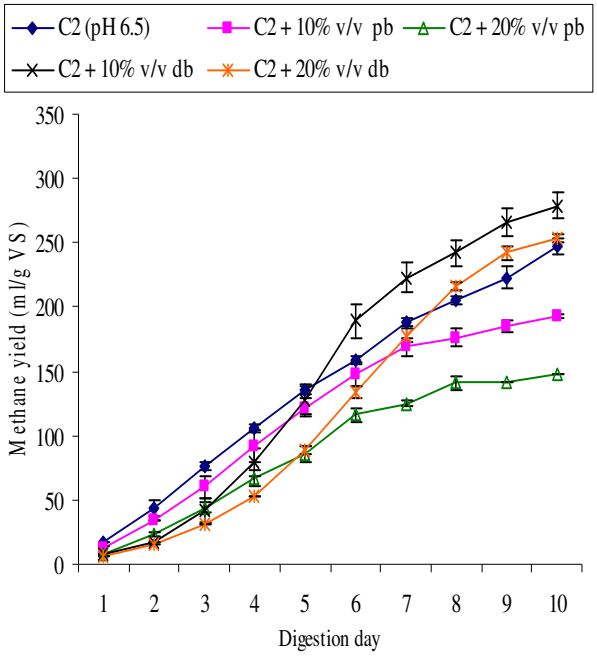
Figures 8.24 (a) and (b). Comparison of methane yields of 10% piggery biomass (pb) and DiCOM biomass (db)-supplemented wastewaters without pH reduction (C1), and comparison of methane yields of 19% piggery biomass (pb) and DiCOM biomass (db)-supplemented wastewaters with pH reduciton (C2) (error bars indicate standard deviations)

With the pH-unadjusted wastewater (C1), while methane yields of both the 10% piggery biomass and 10% DiCOM biomass-supplemented wastewaters were comparable, the methane yield of 19% DiCOM biomass-supplemented wastewater was significantly higher than the 19% piggery biomass-supplemented wastewater at day 11.

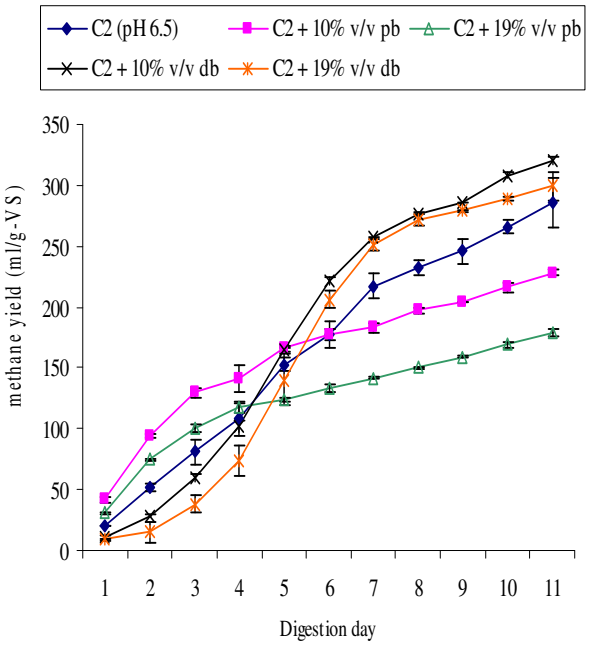
In the case of the pH-reduced wastewater (C2), methane yields of both the 10% and 19% DiCOM biomass-supplemented wastewaters were significantly higher than their counterpart piggery biomass-supplemented wastewaters at day 11 after an initial lag of about 4 days.

As the possibility of biogas leakage through the used rubber stoppers could not be ruled out for the above outcomes particularly with the low methane yield of the piggery biomass, batch digestion experiment on the biomass supplemented-pH reduced wastewater was repeated using new gas-tight rubber stoppers. Figures 8.25 (a) and (b) show the methane yield profiles of the repeat and the earlier experiments respectively. The repeat methane yield profiles were observed to be similar to the earlier profiles of the piggery biomass and DiCOM biomass-supplemented wastewaters, thereby vindicating the earlier findings on higher methane yield of the DiCOM biomass compared to the piggery biomass.

(a) Repeat



(b) Previous

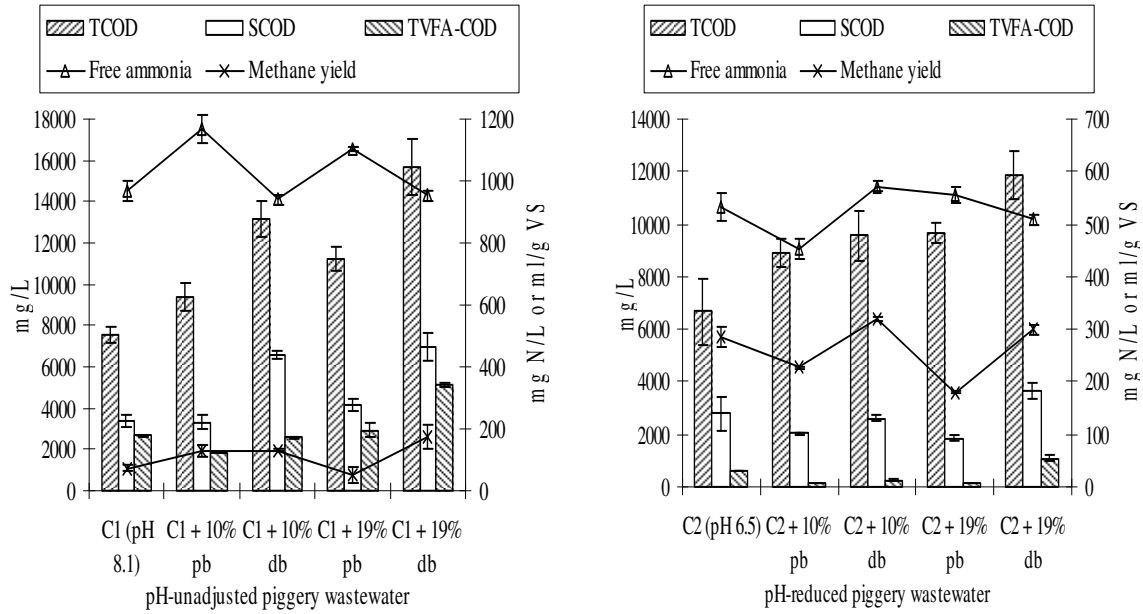


Figures 8.25 (a) Repeat and (b) Previous. Comparison of the methane yields of low (10%) and high (20%) piggery biomass- (pb) and DiCOM (db) biomass-supplemented piggery wastewaters with pH reduction (C2) (error bars indicate standard deviations)

Organic matter (COD, total volatile fatty acids), ammonia-nitrogen and methane yield

Figures 8.26 (a) and (b) illustrate the COD, TVFA, free ammonia and methane yields of the pH-unadjusted and pH-reduced piggery wastewaters supplemented with piggery biomass and DiCOM biomass respectively at the end of the batch digestion period.

(a) (b)



pb (piggery biomass) db (DiCOM biomass)

Figures 8.26 (a) and (b). pH-unadjusted (C1) and pH-reduced (C2) wastewaters' key chemical characteristics at the end of the batch digestion period (error bars indicate standard deviations)

The pH-unadjusted piggery wastewaters supplemented with DiCOM biomass (10% and 19%) were observed to have substantially higher COD and TVFA concentrations at the end of the batch digestion period compared to the control and their counterpart wastewaters supplemented with piggery biomass. The methane yields of 10% and 19% DiCOM biomass-supplemented wastewaters (C1) were comparable with the 10% piggery biomass-supplemented wastewater (Figure 8.26 (a)).

In the case of the pH-reduced piggery wastewaters (C2) supplemented with DiCOM biomass (10% and 19%), while their methane yields were substantially higher than their piggery biomass counterparts and only slightly higher than the pH-reduced control (C2), their COD and TVFA concentrations at the end of the digestion period were significantly higher than the piggery biomass-supplemented wastewaters (Figure 8.26 (b)).

Anaerobic microorganisms

Figure 8.27 shows the distribution of bacteria groups in the pH-unadjusted and pH-reduced piggery wastewaters without and with piggery biomass and DiCOM biomass supplements. The gaps indicate missing sample data.

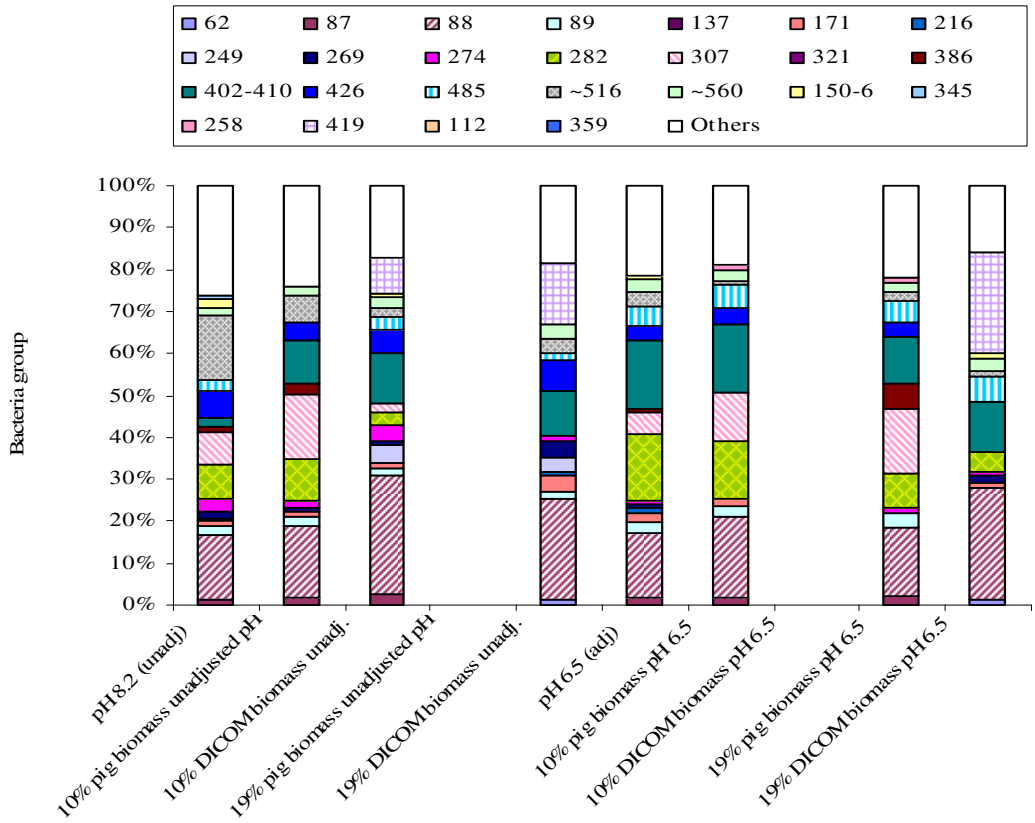


Figure 8.27. Approximate distribution of bacteria group in the controls and biomass-supplemented pH-unadjusted and pH-reduced thermophilic piggery effluent at the end of the test period (day 11)

Addition of biomass (piggery or DiCOM) to the pH-unadjusted piggery wastewater was observed to result in substantially higher levels of 402-410 bp fragment (*Bacteroides, Thermotoga spp., Thermodesulfobacterium*) and lower levels of 516 bp fragment (*Propionibacterium, Actinomycetes* and *Bifidobacterium*). Both pH-unadjusted and pH-reduced piggery wastewaters supplemented with DiCOM biomass had high level of fragment 419 bp (*Prevotella spp./Planctomycetes*) which was absent from the piggery wastewater supplemented with piggery biomass. Appendix 8 gives a list of the bacterial genera that belong to the designated fragment.

8.3.3. Effect of zeolite treatment on methane production from thermophilic batch anaerobic digestion of pH-unadjusted and pH-reduced piggery wastewater

Methane production

Figure 8.28 illustrates the cumulative methane production from the pH-unadjusted (pH 8.1) and pH-reduced (pH 6.5) thermophilic piggery effluent treated with 10, 15 and 20 g/L natural zeolite.

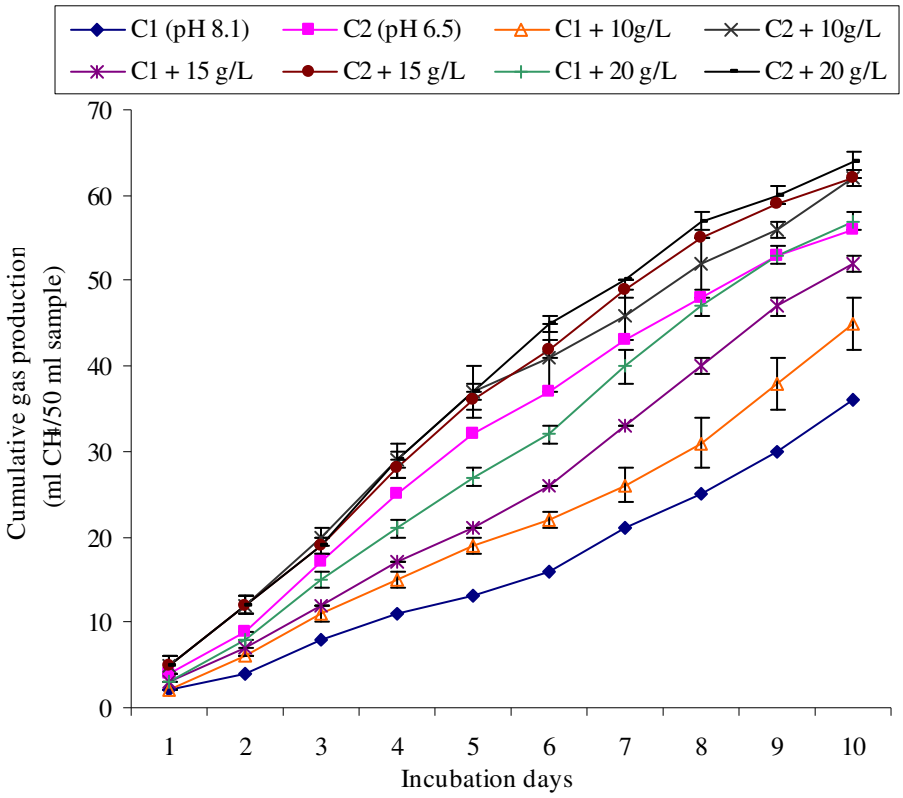


Figure 8.28. Effect of zeolite concentrations on cumulative methane production from thermophilic piggery reactor effluent (error bars indicate standard deviations)

Treatment of the pH-unadjusted piggery effluent (pH 8.1) with 10, 15 and 20 g/L zeolite resulted in significantly higher methane production rates over the control at the end of the digestion period. Highest increase in total methane production rate was observed at 20 g/L zeolite (of 60 ± 4%) which was comparable to the pH-reduced control (57%), followed by 15 g/L and 10 g/L zeolite (of 44 ± 3% and 26 ± 7% respectively).

As observed in previous experiments, pH reduction from 8.1 to 6.5 increased the total methane production by 57% at the end of the digestion period. Treatment of the pH-reduced piggery effluent (pH 6.5) with 10, 15 and 20 g/L zeolite further increased the total methane production but by only 10 ± 1% at 10 and 15 g/L zeolite and 13 ± 0% at 20 g/L zeolite.

Organic reduction (TCOD, SCOD and TVFA-SCOD)

Table 8.13 gives the COD (total and soluble) and total VFA-COD concentrations of the pH-unadjusted (pH 8.1) and pH-reduced (pH 6.5) thermophilic wastewaters at the start and end of the test period. The reductions of wastewater total COD, soluble COD and total VFA-SCOD were graphically presented in Figure 8.29.

Table 8.13. Chemical oxygen demand (total and soluble) and total volatile fatty acids at start and end of the batch serum vial digestion experiment with zeolite treatment

Sample	Day	TCOD (mg/L)	SCOD (mg/L)	TVFA (mg COD/L)
C1 (pH 8.1)	0	8310 (281)	4523 (128)	3240 (125)
C1 (pH 8.2)	11	7157 (225)	3982 (382)	2389 (165)
C1 + 10 g/L	0	7912 (57)	4432 (0)	3387 (142)
C1 + 10 g/L	11	7077 (195)	3387 (62)	1848 (19)
C1 + 15 g/L	0	8628 (0)	4324 (102)	3296 (154)
C1 + 15 g/L	11	7793 (112)	3396 (54)	1546 (146)
C1 + 20 g/L	0	7872 (0)	4144 (255)	3343 (166)
C1 + 20 g/L	11	6600 (210)	3099 (171)	1326 (6)
C2 (pH 6.5)	0	8509 (337)	4342 (127)	2602 (162)
C2 (pH 6.5)	11	6322 (57)	3171 (153)	1462 (78)
C2 + 10 g/L	0	8907 (337)	4487 (179)	2995 (126)
C2 + 10 g/L	11	6521 (325)	2937 (236)	1193 (60)
C2 + 15 g/L	0	8310 (619)	4180 (0)	3130 (104)
C2 + 15 g/L	11	6096 (368)	2811 (216)	1101 (43)
C2 + 20 g/L	0	8628 (168)	4036 (204)	3070 (68)
C2 + 20 g/L	11	6481 (334)	2793 (133)	999 (48)

Data are mean values of replicates (± standard deviation)

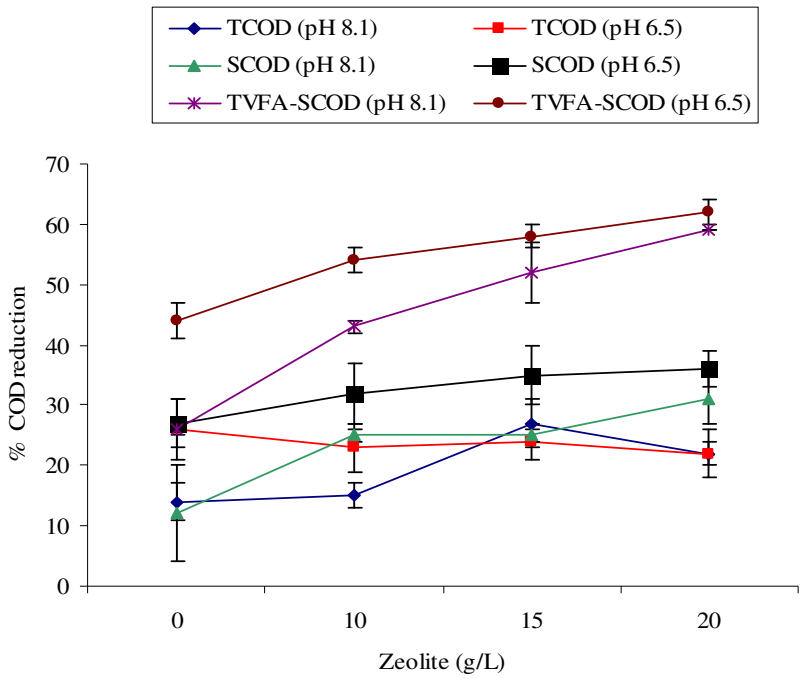


Figure 8.29. COD (total and soluble) and total TVFA reductions in pH-unadjusted (pH 8.1) and pH-reduced (pH 6.6) thermophilic piggery effluent at varying zeolite concentrations (error bars indicate standard deviations) after 10 days of batch digestion

Consistent with the trend in methane production enhancement, pH-unadjusted effluent (pH 8.1) treated with zeolites showed significantly ($p<0.05$) highest total VFA reduction at 20 g/L zeolite, followed by 15 g/L and 10 g/L zeolite over the control. However, no similar corresponding reduction trends in total and soluble COD trends were observed. As explained in previous experiments, sample in-homogeneity and the presence of fine particulates plus non-volatile organic matter were possible factors that had affected the direct COD measurements and distorted the COD reduction data.

In the case of the pH-reduced piggery wastewater (pH 6.5), reduction of total VFA-COD was significantly higher ($p<0.05$) in the effluents with zeolite treatment compared to the control. While there was no significant difference ($p>0.05$) between 10 and 15 g/L doses, there was significant difference ($p<0.05$) between 10 and 20 g/L doses which mirrored their corresponding methane production (Figure 8.28).

In order to determine the relationship between organic COD degradation and zeolite concentration, the apparent kinetic constant of organic COD degradation was calculated using the first-order kinetic model (Milan *et al.,* 2001):

$S/S_0 = \exp - [k_1\ t(X_0/S_0]$ ..... (I)

Where S is the concentration of organic matter (g COD/L) at any digestion time, t (days), S_0 is the initial substrate concentration (g COD/L), k_1 is the first-order kinetic constant ($days^{-1}$) and X_0 is the initial concentration of microorganisms expressed as grams of volatile suspended solids per litre (VSS/L). X_0 and S_0 are assumed constant during the experiment and the product $k_1(X_0/S_0)$ is an apparent constant (k'_1). Simplifying equation 1 yields:

$S/S_0 = (\exp - (k'_1 t)$ ……(2) which is transformed to:

$- \ln (S/S_0) = k'_1 t$ …… (3)

A plot of $- \ln (S/S_0)$ versus t gives a straight line with a slope equal to k'_1 with an intercept at zero. In this study, TVFA-COD data were used as they were specific for volatile organics and were also more consistent than either TCOD or SCOD data. The calculated apparent kinetic constant data are plotted against their corresponding zeolite concentration to determine their correlation coefficient.

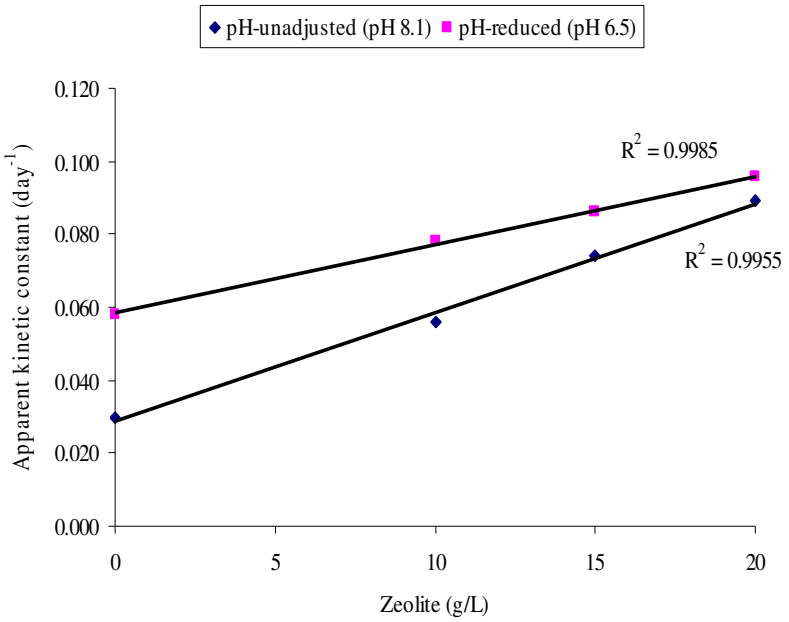


Figure 8.30. Relationship between apparent kinetic constant of TVFA-COD degradation and zeolite concentration at the end of the test period (day 10)

As shown in Figure 8.30, total VFA-COD degradation trends of both the pH-unadjusted (pH 8.1) and pH-reduced (pH 6.5) piggery wastewaters demonstrated strong positive correlation (R^2 = 0.9955 and 0.9985 respectively) with zeolite concentration. This observation indicated that organic degradation rate increased with increased zeolite concentration. However, the converging slopes between zeolite-treated pH-reduced and pH-unadjusted effluents at increasing zeolite concentration suggest that while higher zeolite doses above 20 g/L might not have significant positive influence on the pH-reduced piggery effluent, they could further enhanced the organic removal substantially on the pH-unadjusted piggery effluent.

Volatile fatty acids degradation

Table 8.14 gives the VFA concentrations in the pH-unadjusted and pH-reduced piggery wastewaters treated with zeolite as well as their controls. Figures 8.30 (a) and (b) illustrate the amounts of VFA degraded at varying zeolite concentrations in the thermophilic piggery wastewaters without (C1) and with pH reduction (C2) respectively. Negative percentage reductions indicate VFA accumulation.

Table 8.14. Effect of zeolite on volatile fatty acid concentrations in pH-unadjusted (C1) and pH–reduced (C2) thermophilic piggery wastewaters

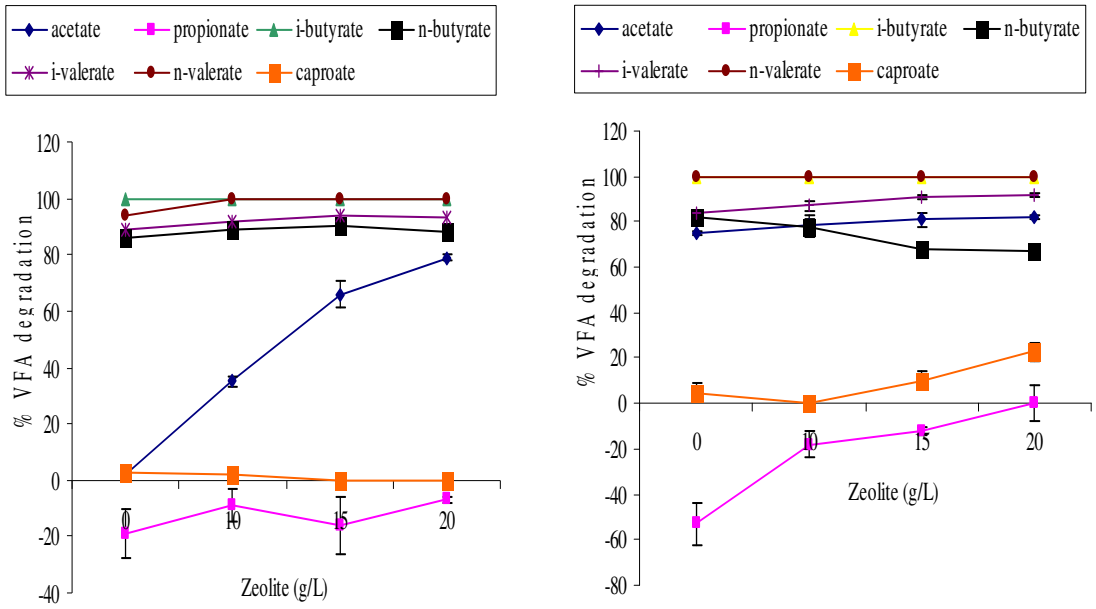
Sample	Day	Acetate	Propionate	i-butyrate	n-butyrate	i-valerate	n-valerate	Caproate
C1 + 0 g/L	0	1119 (51)	871 (34)	232 (9)	140 (8)	571 (30)	200 (9)	108 (16)
C1 + 0 g/L	10	1152 (96)	1037 (79)	0	19 (1)	64 (1)	11 (16)	105 (5)
C1 + 10 g/l	10	728 (24)	950 (55)	0	15 (1)	47 (6)	0	107 (5)
C1 + 15 g/l	10	377 (56)	1011 (89)	0	15 (0)	36 (1)	0	108 (0
C1 + 20 g/l	10	237 (17)	928 (12)	0	16 (0)	39 (0)	0	107 (2)
C2 + 0 g/L	0	802 (99)	705 (47)	224 (12)	114 (6)	485 (26)	184 (4)	88 (0)
C2 + 0 g/L	10	201 (7)	1077 (64)	0	21 (1)	79 (3)	0	84 (3)
C2 + 10 g/l	10	167 (17)	832 (43)	0	25 (5)	63 (9)	0	106 (3)
C2 + 15 g/l	10	156 (26)	788 (13)	0	36 (3)	42 (4)	0	79 (3)
C2 + 20 g/l	10	147 (9)	708 (58)	0	37 (1)	39 (3)	0	68 (3)

All units in mg VFA-COD/L Data are mean values of replicates (± standard deviation)

Without zeolite treatment, both pH-unadjusted (C1) and pH-reduced (C2) wastewaters showed a build-up of propionate while butyrate (i- and n-) and valerate (i- and n-) were either completely or significantly reduced at the end of the digestion

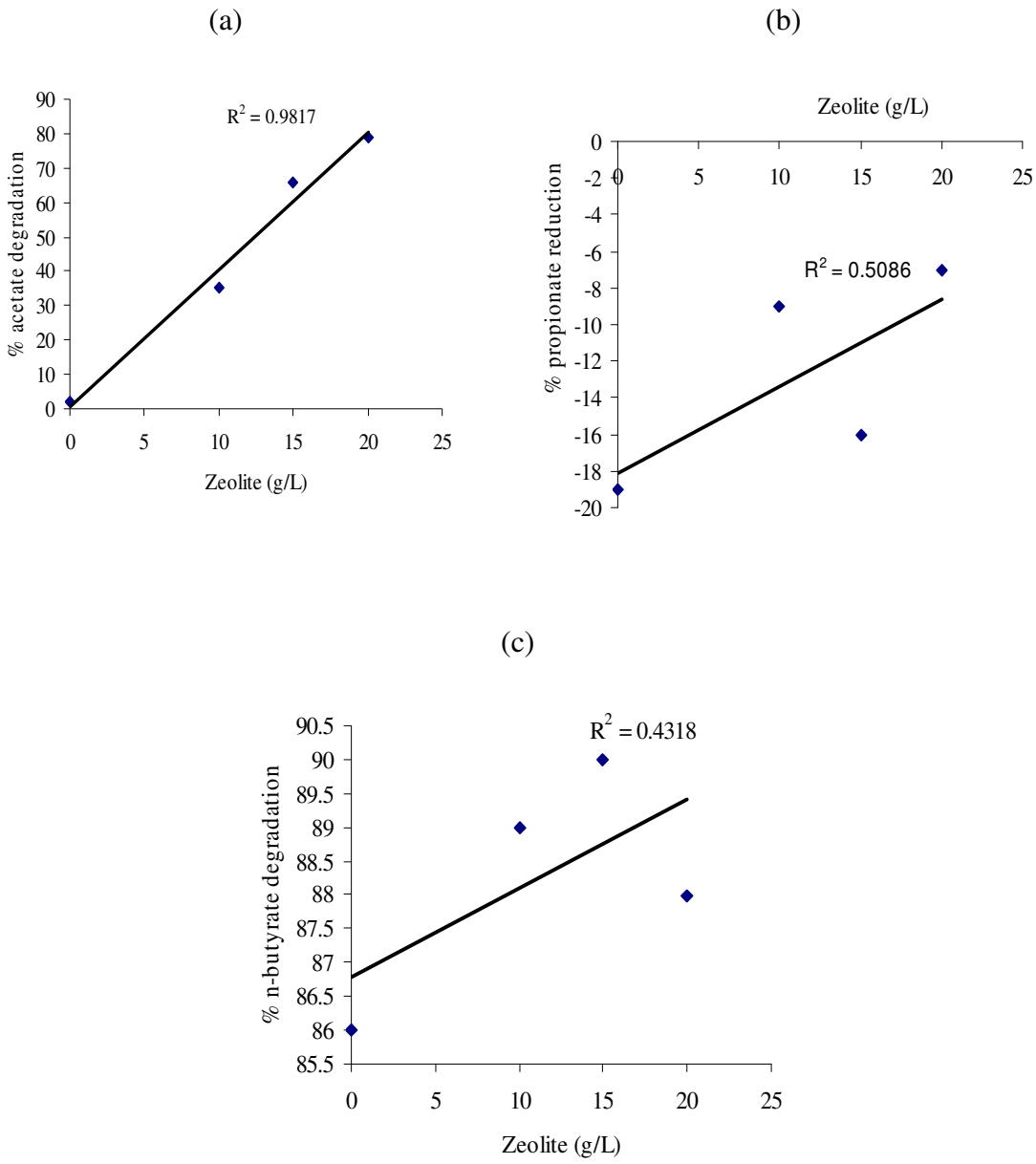
period. Acetate concentration remained relatively unchanged in C1 control in contrast to C2 where acetate degradation was enhanced.

(a) (b)



Figures 8.31 (a) and (b). Effect of zeolite concentrations on VFA degradation in thermophilic piggery effluents without and with pH reduction respectively (error bars indicate standard deviations) after 10 days of batch digestion

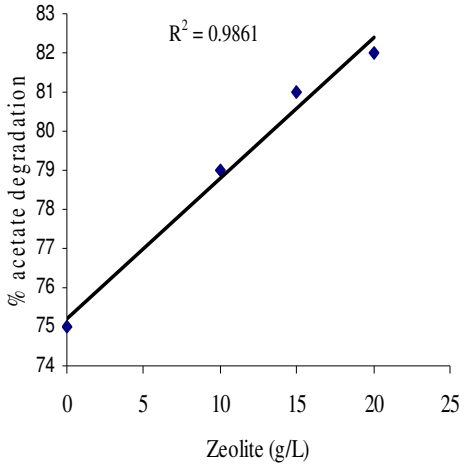
With zeolite-treatment, pH-unadjusted piggery wastewaters showed significant acetate degradation with increasing zeolite doses (Figure 8.31 (a)). Figure 8.32 (a) shows correlation between acetate degradation and zeolite doses was strongly positive (R^2 = 0.9817) which demonstrated the beneficial effect of increased zeolite doses to the acetate-degrading methanogens. Although the zeolite doses applied were observed to be ineffective in reducing the elevated propionate concentration, they promoted further n-butyrate and i-valerate degradation, albeit small in comparison to the control (Table 8.14). Figure 8.32 (b) shows correlation between propionate accumulation and zeolite doses was marginally negative (R^2 = -0.5086) while Figures 8.32 (c) shows correlation between n-butyrate degradation and zeolite doses was weakly positive (R^2 = 0.4318). These observations indicated that the improvements in propionate and n-butyrate degradation were partly contributed by zeolite.



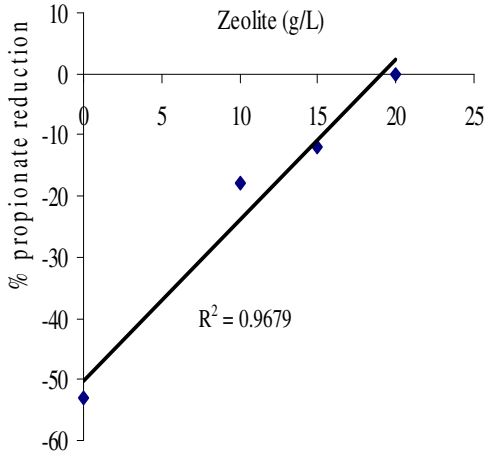
Figures 8.32 (a), (b) and (c). Relationships between acetate degradation, propionate degradation, n-butyrate degradation and zeolite concentrations in the piggery wastewater without pH reduction

Figure 8.31 (b) shows reduction of the piggery wastewater from pH 8.1 to pH 6.5 alone greatly promoted significant enhancement in acetate degradation from 2% to 75%. With zeolite-treatment at 10 to 20 g/L, propionate degradation was greatly improved while small improvements in acetate, i-valerate and caproate degradation were observed over the pH-reduced control (C2).

(a)



(b)



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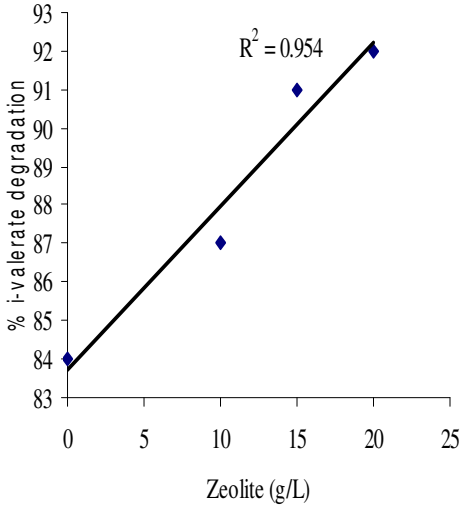
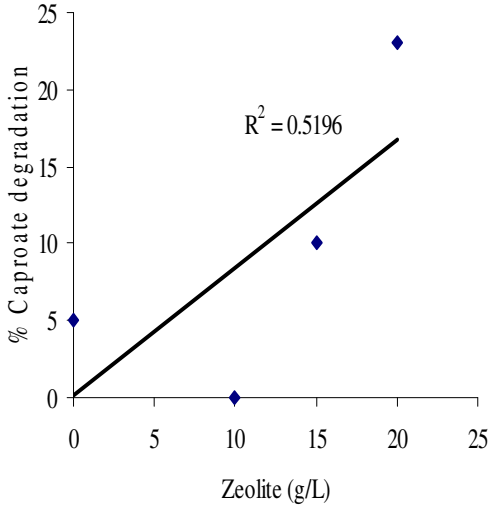


(d)



Figures 8.33 (a), (b), (c) and (d). Relationships between zeolite concentrations and acetate, propionate, i-valerate and caproate degradation in the pH-reduced piggery wastewater

Figures 8.33 (a), (b) and (c) show acetate, propionate and i-valerate degradation displayed strong positive correlation with zeolite doses (R^2 = 0.9861, 0.9679 and 0.954 respectively) while marginal positive correlation (R^2 = 0.5196) was observed between caproate degradation and zeolite doses (Figure 8.33 (d)).

Ammonia, pH and relationships of ammonia with VFA degradation and methane production

Table 8.15 gives the pH, ionised ammonium-nitrogen and unionised dissolved free ammonia concentrations of the pH-unadjusted (C1) and pH-reduced (C2) piggery wastewaters treated with zeolite as well as their controls at the start and end of the digestion period. Without zeolite treatment, both controls showed significant increase in ammonia-nitrogen (ammonium and free ammonia) concentrations and increased pH, in particular the pH-reduced wastewater at the end of the digestion period as a result of microbial degradation of the nitrogen-containing proteins and urea.

Table 8.15. pH, ammonium-nitrogen and dissolved free ammonia concentrations in pH-unadjusted (C1) and pH-reduced (C2) digested piggery wastewaters without and with zeolite treatment

Sample	Day	pH	NH_4^+-N (mg/L)	Free NH_3 (mg/L)
C1 + 0 g/L	0	8.1 (0)	1740 (11)	569 (4)
C1 + 0 g/L	10	8.2 (0)	2015 (15)	765 (6)
C1 + 10 g/L	10	8.3 (0)	1952 (48)	850 (21)
C1 + 15 g/L	10	8.3 (0)	2165 (41)	942 (18)
C1 + 20 g/L	10	8.3 (0)	1908 (11)	830 (5)
C2 + 0 g/L	0	6.5 (0)	1721 (6)	26 (0)
C2 + 0 g/L	10	7.9 (0)	1830 (13)	430 (3)
C2 + 10 g/L	10	7.8 (0)	2017 (290)	395 (57)
C2 + 15 g/L	10	7.8 (0)	1988 (102)	389 (20)
C2 + 20 g/L	10	7.8 (0)	1934 (22)	379 (4)

Data are mean values of replicates (± standard deviation)

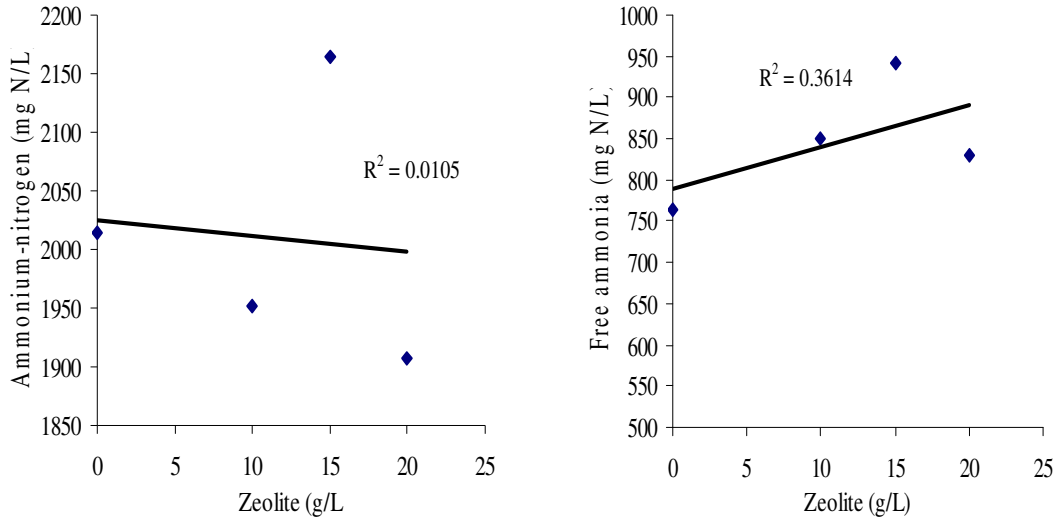
With zeolite-treatment of the pH-unadjusted piggery wastewater, it was observed that both the ammonium-nitrogen and free ammonia concentrations were still high in comparison to the control at the end of the digestion. While the final ammonium-nitrogen concentrations at 20 g/L zeolite was significantly ($p<0.05$) less than the control, its final dissolved free ammonia concentrations were significantly higher compared to the control as a result of a 0.1 pH unit increase.

As pH is one of the variables that influenced the free ammonia level, pH sensitivity test was undertaken on the free ammonia equation given in section 6.2.1.2 of Chapter

6 to determine free ammonia variation for a 0.1 pH unit difference. Reduction of the zeolite-treated wastewater pH by 0.1 unit to the control wastewater pH of 8.2 resulted in a 13% decrease in free ammonia concentrations of zeolite-treated wastewaters (10, 15 and 20 g/L) to 741 ± 18, 822 ± 16 and 725 ± 4 mg N/L respectively. These values were still relatively high in comparison to the control (765 ± 6 mg N/L) and highlighted that the zeolite doses applied were inadequate for effective reduction of the high ammonium ions through ion-exchange.

Figure 8.34 (a) shows there was no correlation between ammonium-nitrogen and the zeolite doses (R^2 = 0.0105). The weak positive correlation (R^2 = 0.3624) between free ammonia concentrations and zeolite doses (Figure 8.34 (b)) reinforced the fact that the zeolite doses applied were not high enough to adsorb the bulk of the ammonium ions in the piggery wastewaters. It was very likely that the zeolite applied were fully saturated with the adsorbed ammonium ions.

(a) (b)

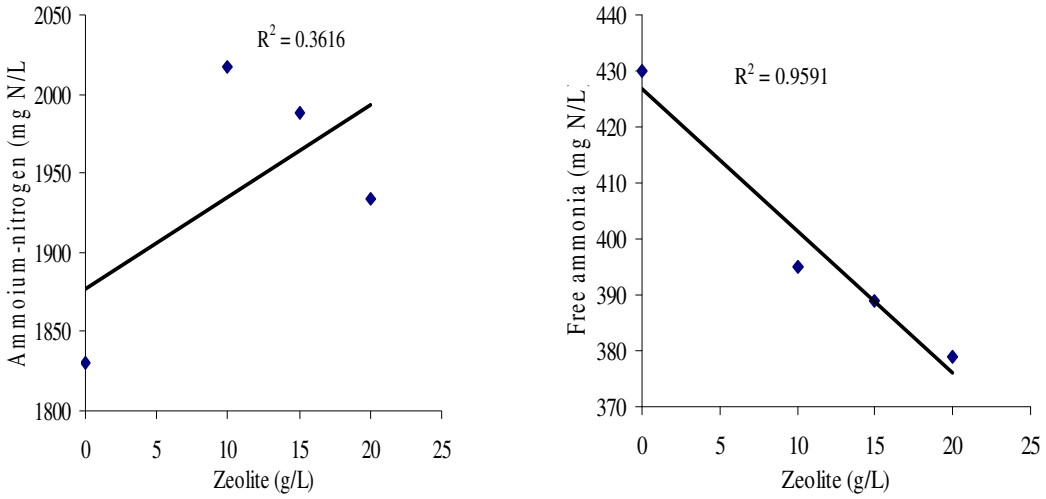


Figures 8.34 (a) and (b). Relationships between ammonium-nitrogen and zeolite concentrations in the thermophilic piggery wastewater without pH reduction; and between free ammonia and zeolite concentrations in the thermophilic piggery wastewater without pH reduction respectively

Reducing the piggery wastewater pH from its initial pH of 8.1 to pH 6.5 significantly lowered the initial dissolved free ammonia concentration by 95% at 55°C. Similar to the pH-unadjusted wastewater treated with zeolite, the ammonium-nitrogen and free ammonia concentrations were still high in comparison to the control at the end of the

digestion. While the final free ammonia concentrations at 20 g/L zeolite was significantly ($p<0.05$) less than the control at the measured pH of 7.8 (379 ± 4 mg N/L), pH sensitivity test at the control pH of 7.9 showed that a measurement error of 0.1 pH unit would increase the free ammonia concentration by 20% to 454 ± 5 mg N/L which would make it significantly ($p<0.05$) higher than the control (430 ± 3 mg N/L). Irrespective of the true values, the final ammonium-nitrogen and free ammonia concentrations of the pH-reduced wastewaters treated with zeolite were still relatively high compared to the control, again highlighting the inadequacy of zeolite doses applied for effective removal of the high ammonia-nitrogen concentration in the wastewater.

(a) (b)



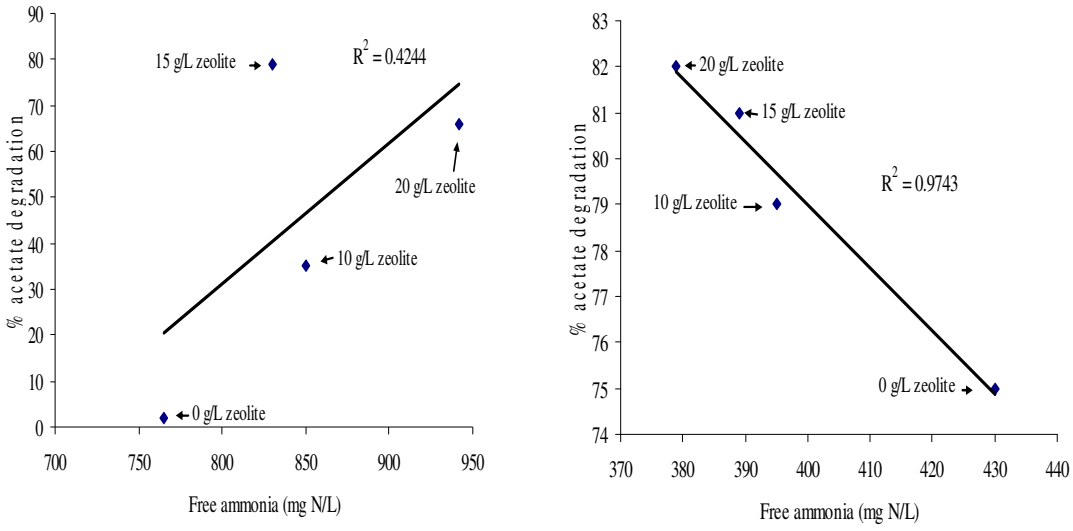
Figures 8.35 (a) and (b). Relationships between ammonium-nitrogen and zeolite concentrations in the pH-reduced piggery wastewater; and between free ammonia and zeolite concentrations in the pH-reduced piggery wastewater respectively

Figures 8.35 (a) and (b) illustrate the relationships between ammonium-nitrogen or free ammonia nitrogen and zeolite concentrations. While correlation between free ammonia concentrations and zeolite doses was strongly negative (R^2 = -0.9591) which indicated greater reduction of the unionised free ammonia with higher zeolite doses, the ammonium-nitrogen concentrations showed weak positive correlation (R^2 = 0.3616) with the zeolite doses. This reinforced the notion that the zeolite doses applied were not high enough to adsorb the bulk of the ammonium ions in the

piggery wastewaters. It was very likely that the zeolite doses applied were fully saturated with the adsorbed ammonium ions.

As unionised free ammonia is toxic to methane-forming microorganisms at high concentration and increases with pH and temperature (Figure 2.4 in Chapter 2), attention is focused here on its relationship with acetate which is the main VFA precursor for methane production. Figure 8.36 (a) shows a weak positive correlation (R^2 = 0.4244) between free ammonia concentrations and acetate degradation, which implied that the improvement in acetate degradation with zeolite in the pH-unadjusted wastewater was not attributed to ammonia reduction mechanism of the zeolite. It is possible that the enhanced acetate degradation observed was due to other zeolite mechanisms such as the immobilisation of acetoclastic methanogens within the channels of the zeolite and the combined microbial stimulation by unknown zeolite cations released during ion-exchange with the wastewater ammonium ions.

(a) (b)



Figures 8.36 (a) and (b). Relationships between acetate degradation and free ammonia concentrations in the thermophilic piggery wastewaters without pH-reduction (C1) and with pH-reduction (C2) respectively

In the case of the pH-reduced wastewater (C2), Figure 8.36 (b) shows strong negative correlation (R^2 = -0.9743) between acetate degradation and free ammonia concentrations. This implies that the improvement in acetate degradation could not

be attributed to ammonia adsorption mechanism of the zeolite as the ammonium-nitrogen concentrations were not reduced in the zeolite-treated wastewater. It is possible that a combination of free ammonia reduction by the pH reduction method and the aforementioned zeolites mechanisms of microbial immobilisation and stimulation by the released unknown zeolite cations could have contributed to the improvement in acetate degradation.

Anaerobic microorganisms

Real-time PCR analysis of the methanogen population found pH-reduced piggery wastewater treated with zeolite had greater number of methanogens (1.2 x 10^6 cells/ml) compared to the pH-unadjusted piggery wastewater (1.1 x 10^5 cells/ml). Figure 8.37 shows an approximate distribution of the type of bacteria group in the control and the pH-unadjusted and pH-reduced wastewaters treated with zeolite. The gap indicates missing sample data.

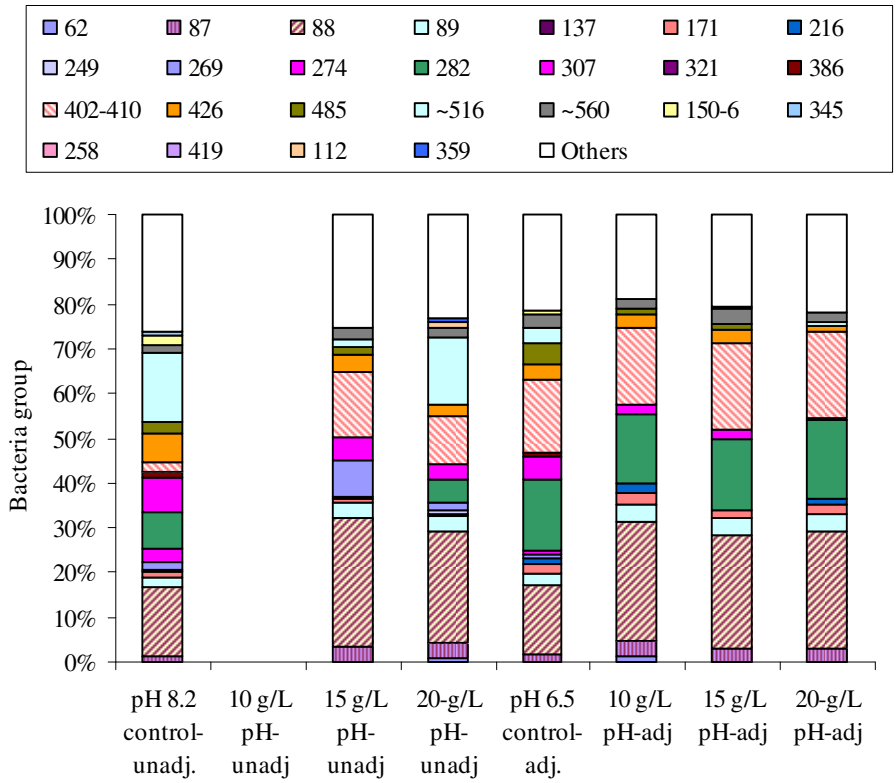


Figure 8.37. Approximate distribution of bacteria group in the controls and zeolite-treated pH-unadjusted and pH-reduced thermophilic piggery wastewaters at the end of the test period (day 10)

The profile of pH-unadjusted wastewater treated with zeolite showed an increased in fragment 89 bp (included propionate-producers *Viollenella, Megasphaera*) and fragment 402-410 bp (included *Bacteroides, Thermotoga spp., Thermodesulfobacterium*) whilst fragments 485 (*Cytophaga, Bacteriodes*) and 274 (*Myxococcus, Capnoctophaga, Clostridium*) decreased compared to the control. In the case of the pH-reduced wastewater treated with zeolite, an increase in the proportion of 402-410 fragment (*Bacteroides, Thermotoga spp., Thermodesulfobacterium*) and 89 bp (included propionate-producers *Viollenella, Megasphaera*) were observed. Appendix 8 gives a list of the bacterial genera that belong to the designated fragment.

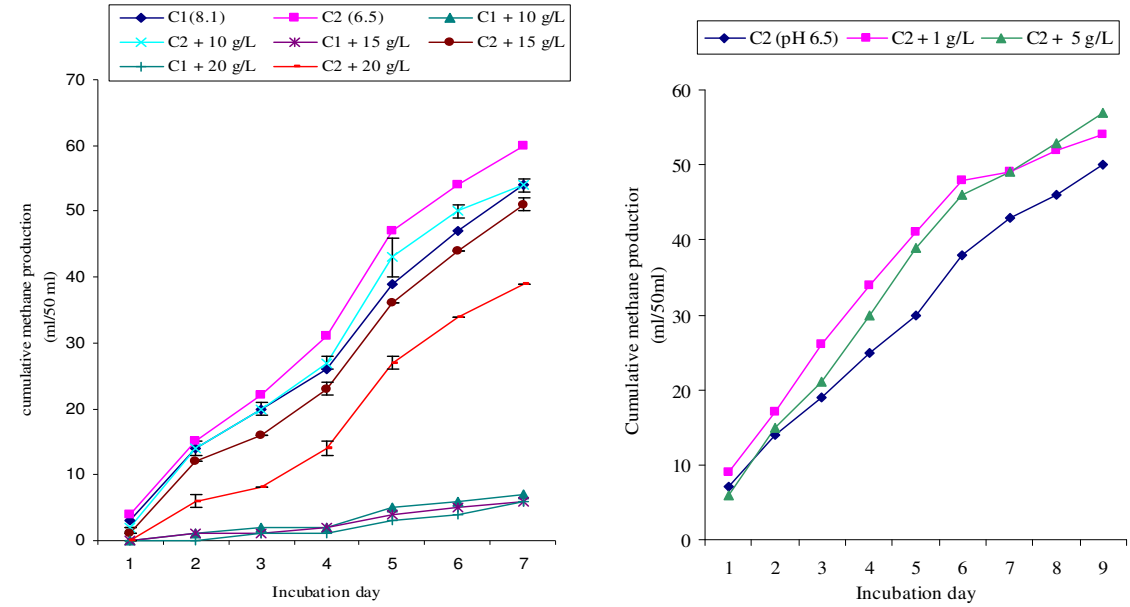
8.3.4. Effect of humic acid supplements on methane production from thermophilic batch anaerobic digestion of pH-unadjusted and pH-reduced piggery effluent

Methane production

Figures 8.38 (a) and (b) illustrate the cumulative methane production of the pH-unadjusted (pH 8.1) and pH-reduced (pH 6.5) thermophilic piggery wastewaters with high doses (10, 15 and 20 g/L) and low doses (1 and 5 g/L) of humic acid added respectively.

At 10, 15 and 20 g/L of humic acid, methane production rates were greatly reduced which suggested that the methanogens were severely inhibited, particularly in the pH-unadjusted effluent (C1). It was observed that at these concentrations, the piggery wastewater became increasingly viscous and blackish in appearance. No further chemical analysis were conducted on these samples. At low concentrations of 1 and 5 g/L, small improvements of 27-29% in methane production rates were observed during the first 6 days compared to the control (C2).

(a) (b)



Figures 8.38 (a) and (b). Effect of humic acid (high) on methane production of thermophilic piggery effluents without pH-reduction (C1) and pH-reduction (C2); ad effect of humic acid (low) on methane production of pH-reduced (C2) thermophilic piggery effluent respectively (error bars indicate standard deviations)

Organic reduction (TCOD, SCOD and TVFA-SCOD)

Table 8.16 gives the COD (total and soluble) and total VFA-COD concentrations of the pH-reduced (pH 6.5) thermophilic wastewater at the start and end of the test period. The reductions of wastewater total COD, soluble COD and total VFA-SCOD were graphically presented in Figure 8.39.

Table 8.16. Chemical oxygen demand (total and soluble) and total VFA of the thermophilic piggery wastewater at start and end of the batch serum vial digestion experiment

Sample	Day	TCOD (mg/L)	SCOD (mg/L)	TVFA (mg COD/L)
C2 (pH 8.1)	0	8168 (169)	4739 (284)	2667 (209)
C2 (pH 8.1)	9	6096 (56)	3233 (142)	1515 (10)
C2 + 1 g/L	0	9203 (395)	4639 (29)	2823 (13)
C2 + 1 g/L	9	7371 (169)	3233 (426)	1069 (93)
C2 + 5 g/L	0	13864 (0)	9421 (251)	3528 (106)
C2 + 5 g/L	9	12350 (338)	5341 (284)	1517 (51)

Data are mean values of replicates (± standard deviation)

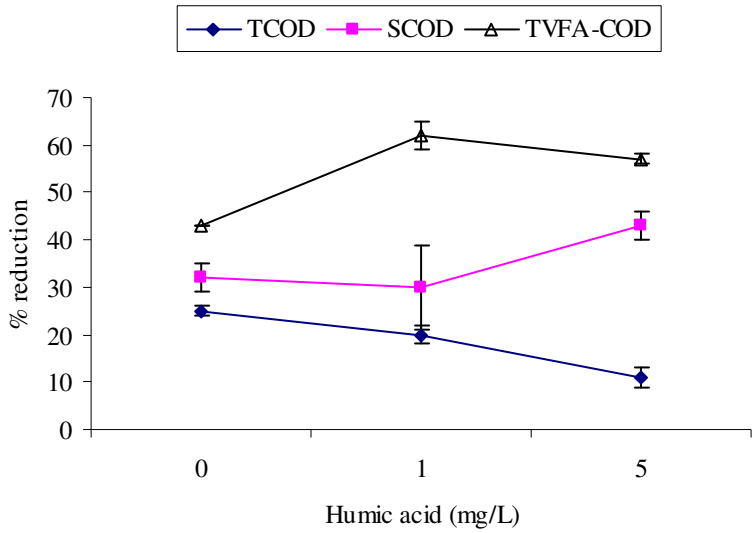


Figure 8.39. COD (total and soluble) and total TVFA reductions in pH-reduced (pH 6.5) thermophilic piggery effluent at low humic acid concentrations (error bars indicate standard deviations)

Additions of 1 and 5 g/L humic acid were found to raise the initial and final total COD concentrations of the piggery wastewater while 5 g/L humic acid also raised the initial and final soluble COD concentrations as well as the initial TVFA concentration substantially (Table 8.16). Inconsistencies in reduction trends were observed between TVFA-SCOD and COD particularly TCOD (Figure 8.39) which could be due largely to interference by the insoluble impurities present in the technical grade humic acid. Reduction of TVFA-COD was marginally higher at the lower dose of 1 g/L (62 ± 3%) than 5 g/L (57 ± 1%) humic acid-treated wastewater.

Volatile fatty acids degradation

Table 8.17 gives the VFA concentrations of the pH-reduced piggery wastewaters treated with humic acid and the control while Figure 8.40 illustrates the amount of VFA degraded.

Table 8.17. Effect of zeolite on volatile fatty acid concentrations in pH-unadjusted (C1) and pH–reduced (C2) thermophilic piggery wastewaters

Sample	Day	Acetate	Propionate	i-butyrate	n-butyrate	i-valerate	n-valerate	Caproate
C2 (pH 6.5)	0	705 (65)	573 (48)	120 (5)	55 (4)	232 (13)	82 (6)	43 (5)
C2 (pH 6.5)	9	225 (7)	740 (3)	0	0	33 (0)	0	41 (1)
C2 + 1 g/L	0	807 (23)	594 (1)	104 (5)	62 (2)	235 (2)	96 (1)	43 (1)
C2 + 1 g/L	9	131 (21)	537 (36)	0	0	21 (4)	0	35 (4)
C2 + 5 g/L	0	1015 (29)	764 (19)	120 (6)	95 (6)	277 (13)	121 (8)	42 (3)
C2 + 5 g/L	9	305 (19)	617 (16)	0	0	78 (4)	0	46 (0)

Data are mean values of replicates (± standard deviation)

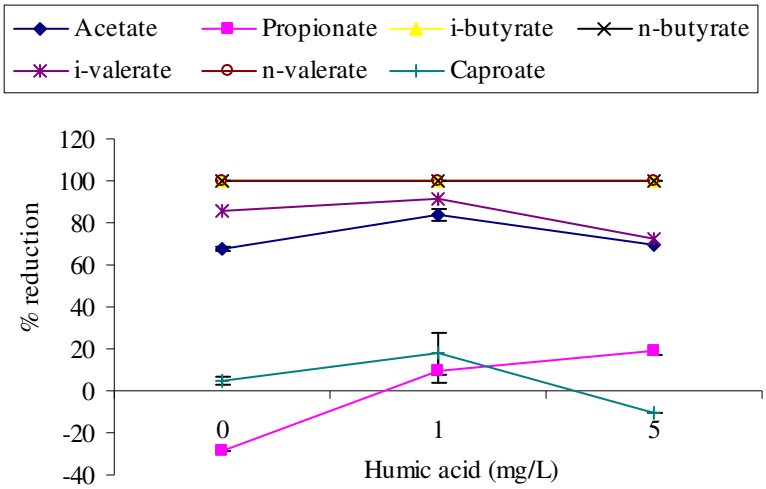


Figure 8.40. Effect of humic acid concentrations on VFA degradation in pH-reduced thermophilic piggery effluent (error bars indicate standard deviations)

Without humic acid treatment, the control (C2) showed a build-up of propionate while acetate, butyrate (i- and n-) and valerate (i- and n-) were either completely or significantly reduced at the end of the digestion period. At 1 and 5 g/L humic acid treatment, significant enhancement in propionate degradation over the control was observed. Small improvements in acetate, i-valerate and caproate degradation were also observed at the lower dose of 1 g/L (Figure 8.40).

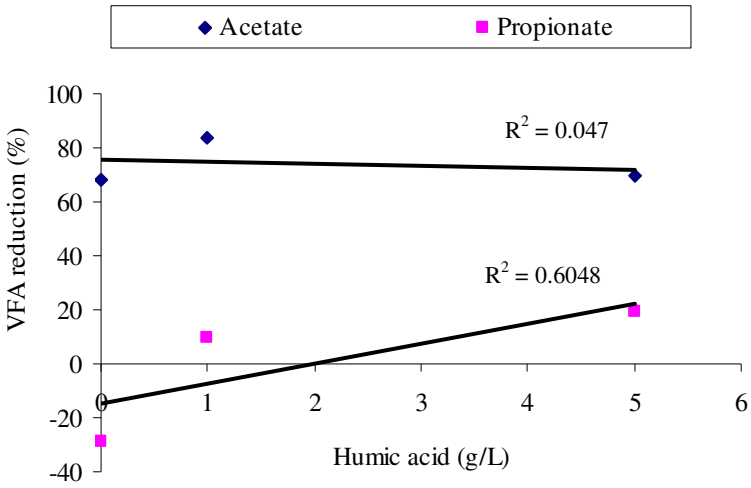


Figure 8.41. Relationships between humic acid concentration and VFA degradation (acetate and propionate) in the pH-reduced piggery wastewater

Figure 8.41 shows that while there was no relationship between acetate degradation and humic acid doses (R^2 = 0.047), propionate degradation showed marginal positive correlation (R^2 = 0.6048) with humic acid doses which suggested that the high hydrogen concentration which inhibited the propionate degradation was partially reduced by the humic acid serving as electron acceptors.

8.4. DISCUSSION

Although digested piggery effluent from 10-day HRT reactor experiment was used in each of the five batch experiments below, it should be noted that the variations observed in pH and COD concentrations (total, soluble, TVFA) of the controls among the different experiments were attributed to a combination of factors which included the inhomogenenous nature of the piggery effluent collected in different bottles, different initial treatment of the effluents, different starting time of the experiments and errors incurred in sampling and analysis.

8.4.1. Effect of pH on methane production from thermophilic batch digestion of piggery effluent

The results from this study clearly demonstrated the effectiveness of pH reduction of the slightly alkaline piggery effluent from its initial pH of 8.3 to pH 6.5 in reducing

the effluent high free ammonia concentration. This resulted in enhanced reductions in COD, VFA in particular acetate and propionate as well as enhanced methane production at the lower pH of 6.5. The results highlighted the importance of providing an optimum pH for the mixed anaerobic microorganisms to function efficiently in the anaerobic digesters. The optimal pH required for acidogenic bacteria is reported to be between 5.0 and 6.5 while the optimal pH for acetogens and methanogens is at approximately pH 7 (Angelidaki *et al*., 2003; Hall, 1992). It was also reported that at pH lower than 6.3 and higher than 7.8, the rate of methanogenesis decreases (van Haandel *et al*., 2006).

Ammonia is a product of urea hydrolysis or anaerobic protein degradation (Gallert *et al*., 1998). It is essential for microbial growth but at high concentration, it inhibits methanogenesis. Free ammonia is widely known as the active component of the total ammonia that causes ammonia inhibition. The unionised molecule is lipophilic and diffuses rapidly through majority of the biological membrane, disrupting the intracellular metabolism (Gerardi, 2006; Gallert and Winter, 1997; Kleiner *et al.*, 1998). Its concentration depends mainly on the total ammonia concentration, temperature and pH with a decrease in pH or temperature having the effect of decreasing the free ammonia concentration (Figure 2.4 in Chapter 2). The high positive correlation coefficient value ($R^2 = 0.9401$) observed between the wastewater initial pH and its final free ammonia concentration (Figure 8.9) demonstrated their strong positive relationship and the effectiveness of pH reduction in lowering the wastewater's high ammonia concentration (916 ± 32 mg N/L) at pH 8.3 to a low concentration (24 ± 0 mg N/L) at pH 6.5.

The high final free ammonia levels (643 ± 50 to 853 ± 101 mg N/L) observed at the initial pH of 7, 7.5 and 8.3 (Table 8.3) were close to or above the inhibitory value of 700 mg-N/L reported by Angelidaki and Ahring (1994) at pH 7.4-7.9 and way above 560-568 mg-N/L reported by Gallert and Winter (1997) to cause 50% inhibition of methanogenesis at pH 7.6. The strong negative correlations between free ammonia levels and acetate degradation as well as propionate degradation (Figure 8.11) implicated the high free ammonia levels to have inhibited the acetate-utilising methanogens as well as the syntrophic propionate-degrading acetogenic bacteria and

hydrogenotrphic methanogens at initial pH above 6.5, resulting in the accumulation of acetate and propionate (Table 8.2).

Propionate in particular, is well known for being the most difficult VFA to degrade. Its degradation is thermodynamically possible only when hydrogen, the reduced product of VFA oxidations is maintained at a very low concentration ($<10^{-4}$ atm) by the hydrogen-utilising microorganisms (Harper and Pohland, 1987; Thauer *et al*., 1977). The elevated propionate concentrations at initial pHs above 6.5 in this study thus indicated that the hydrogen-utilising microorganisms had been inhibited by the high ammonia concentrations in the piggery effluents. This observation concurred with Wiegant and Zeeman (1986) who found propionate to accumulate when the hydrogen-utilising methanogens were inhibited by high ammonia concentration. It is postulated that ammonia inhibition of hydrogen-utilising microorganisms resulted in the build-up of hydrogen partial pressure which inhibited the syntrophic propionate-degrading acetogenic bacteria from converting propionate to acetate, hydrogen and carbon dioxide. The outcome of hydrogen inhibition was the accumulation of propionate (Table 2.2 in Chapter 2). The accumulated hydrogen and propionate in conjunction with high free ammonia level could have combined to inhibit the acetoclastic methanogens from converting the acetate to methane and carbon dioxide, therefore resulting in acetate accumulation.

Besides ammonia, intermediate products such as hydrogen, propionate and acetate have been reported to inhibit acetate degradation while hydrogen and acetate inhibited propionate degradation (Wiegant and Zeeman, 1986). The inhibitory effects of acetate on acetate and propionate degradation were clearly shown by the strong negative correlations between them (Figures 8.6 (a) and (b)). Unlike propionate, butyrate is reportedly less sensitive to hydrogen concentration and it can be degraded at hydrogen concentration up to 5-6 times higher than the concentration required for propionate degradation (Pind *et al.*, 2003). The higher tolerance of butyrate-degrading bacteria to hydrogen was confirmed by this study's findings of unimpeded butyrate and valerate degradation while propionate degradation was inhibited (Figure 8.5). Calli *et al.* (2005) have also reported high butyrate degradation while propionate degradation was inhibited by free ammonia concentration above 200 mg/L.

The observation that pathogenic *Clostridum perfringens* population was significantly reduced while the methanogenic population showed concurrent increase with increased methane production when the piggery effluent pH was reduced from 8.3 to 6.5 (Skillman *et al.*, 2009) suggested that the pH-altered environment had preferentially promoted the optimum growth of methanogens over the *Clostridum perfringens.* However, the exact cause of the lower number of *Clostridum perfringens* was unclear although microbial competition for common substrates probably played a role in combination with other factors known to cause pathogens decay during treatment of biowastes. These include temperature, retention period, pH and chemical interactions (Salsali *et al.*, 2008; Smith *et al.,* 2005).

While this batch vial experiment clearly demonstrated the stimulatory effect of pH reduction on methane production, it is important to note the practical problems associated with this biogas enhancement approach. As the piggery wastewater has an inherent high buffering capacity, it thus has a tendency to foam and spill over as its pH was reduced to 6.5 with concentrated hydrochloric acid. The foaming was observed to be less severe than in the first-stage anaerobic reactor experiment where the effluent pH was reduced to pH 5.5 in an attempt to inhibit the methanogenic microorganisms. The use of highly corrosive concentrated hydrochloric acid for pH reduction will require utmost safety precautions to be taken to safeguard personnel health and safety.

8.4.2. Effect of biomass supplements on methane production from thermophilic batch anaerobic digestion of pH-unadjusted and pH-reduced piggery effluent

Supplementing biomass to the reactor wastewater was akin to the concept of a contact CSTR configuration where settled biomass was recycled back to the reactor to promote further biodegradation of wastewater particulate organic matter (Hall, 1992). The review paper of Yadvika *et al*. (2004) documented a few studies which reported enhanced biogas production with this approach.

In this study, it was demonstrated that supplementing 10% piggery or DiCOM biomass to the piggery effluent without pH reduction (C1) was more effective than supplementing 19% biomass in enhancing TVFA reduction and methane production. Supplementing higher biomass concentration of 19% resulted in higher initial and

final acetate, propionate and free ammonia concentrations in the wastewaters compared to the control (C1) and 10% biomass supplemented wastewater. These elevated components were known to inhibit methanogenesis as mentioned in the earlier batch pH effect experiment (section 8.4.1). Hydrogen sulphide, another known inhibitor of methanogenesis (Hansen *et al.*, 1999) which was not measured in this study could also have contributed to the combined product inhibition on the microbial consortia of hydrogen-utilisers, propionate-degraders and acetoclastic methanogens at higher biomass supplement (19%). Both the piggery biomass- and DiCOM biomass-supplemented piggery wastewaters were observed to have higher levels of sulphate-reducing bacteria (*Thermodesulfobacteria*) by T-RFLP microbial profiling than the control.

While the methane yield of the pH-unadjusted wastewater with 10% piggery biomass supplement was higher than the pH-unadjusted control (C1), its yield was still two-fold lower than the pH-reduced control (C2) as shown in Figure 8.14. It is likely that its higher initial free ammonia level and non-optimum pH (Table 8.7) had inhibited the activities of the syntrophic acetogens and methanogens. Unlike the piggery biomass, DiCOM biomass (10% and 19%) was observed to require 3 to 5 days to get acclimatised to the pH-uuadjusted and pH-reduced piggery wastewaters before its methane production exceeded the control wastewater (Figures 8.19).

Supplementing piggery biomass (10% and 19%) to the piggery wastewaters with pH-reduction (C2) substantially increased SCOD and TFVA reductions. However, they appeared to suffer from substrate limitation after 3 days of batch digestion as evidenced by their substantially lower final TVFA concentrations and decreasing methane yields compared to the control (C2) due possibly to higher microbial activities (Figure 8.14). In contrast, supplementing 10% DiCOM biomass to the pH-reduced wastewater greatly enhanced propionate degradation and slightly increased methane yield over the control as well as the 19% DiCOM biomass-supplemented piggery wastewater.

Although the DiCOM biomass (10% and 19%)-supplemented piggery wastewaters with pH reduction had substantially higher methane yields than its piggery biomass-supplemented counterparts, it was observed that the addition of DiCOM biomass to the pH reduced wastewater greatly elevated its initial COD and TVFA concentrations

(Tables 8.9). This observation suggested that the greatly enhanced methane yields by the DiCOM biomass supplements were due to higher amount of VFA substrates present in the DiCOM biomass which were available for microbial degradation.

In view of the substantially higher final TCOD concentrations in the digested wastewaters supplemented with biomass (DiCOM or piggery) compared to the pH-unadjusted (C1) and pH-reduced (C2) controls, it is concluded that pH reduction alone was a better option than supplementing DiCOM or piggery biomass to the piggery wastewaters for methane production enhancement.

8.4.3. Effect of zeolite treatment on methane production from thermophilic batch anaerobic digestion of pH-unadjusted and pH-reduced piggery effluent

The results from this study demonstrated the enhancement effect of 10 to 20 g/L zeolite concentrations in total VFA reduction and methane production from pH-unadjusted and pH-reduced piggery wastewaters, with 20 g/L zeolite promoting the highest enhancement effect. The enhancement effect of zeolite doses was greater on the pH-unadjusted piggery wastewater (pH 8.2) than on the pH-reduced wastewater (pH 6.5), due probably to a change of NH_4^+-NH_3 equilibrium with pH (Gerardi, 2003; Milan *et al*, 2001; section 2.5.4 of Chapter 2) and/or ion-exchange competition between H^+ and NH_4^+ cations of the pH-reduced wastewater. The improvement particularly in propionate degradation in the pH-reduced wastewater (Figure 8.31 (b)) was indicative of a reduction in dissolved hydrogen concentration or partial pressure which at level above 10^{-4} atm inhibits propionate degradation (section 2.5.7 of Chapter 2).

These stimulatory zeolite concentrations observed in this study were higher than the optimum concentrations of 2- 4 g/L and 8 g/L reported by Milan *et al.* (2001) and Kotsopoulos *et al.* (2008) to enhance mesophilic and thermophilic anaerobic digestion of swine manure wastewater respectively. Beyond these optimum concentrations, they observed methane production to decrease. A notable difference observed in the swine wastewater characteristics of Milan *et al.* (2001), Kotsopoulos *et al.* (2008) and this study was the substantially lower ammonium-nitrogen concentrations in Milan *et al.* (410 mg NH_4^+-N/L) and Kotsopoulos *et al.* (275 mg NH_4^+-N/L) compared to this study (1740 mg NH_4^+-N/L). For high ammonium-

enriched sludge (4500 mg-N/L), Tada *et al*. (2005) observed three-fold increase in methane production with 5% (50 g/L) and 10% (100 g/L) natural mordenite zeolite doses but at 20% (200 g/L), methane production decreased significantly.

In all these studies, the final ammonium-nitrogen concentrations of the zeolite-treated wastewaters remained high relative to their controls. Milan *et al.* (2001) also observed increasing wastewater's ammonium-nitrogen concentration with increasing zeolite concentrations of 0.2 to 10 g/L. The increase coincided with a decrease in the wastewater organic nitrogen concentration and an increase in ammonium-nitrogen concentration on the zeolites. Kotsopoulos *et al.* (2008) reported slight reduction of wastewater's ammonium-nitrogen concentration with 4 to 12 g/L zeolite but increased free ammonia concentration with increased pH. In a comparative study of methane enhancement effect using different inorganic natural and synthetic zeolites, Tada *et al.* (2005) reported that some natural zeolites which included clinoptilolite zeolite did not enhance methane production while the synthetic zeolites depressed methane production although they removed similar amount of ammonium-nitrogen as the best performing natural mordenite zeolite that produced highest enhancement effect on methane production. The observed improvement by mordenite zeolite was attributed to the elevated calcium concentration released from cation-exchange with the calcium ions of mordenite zeolite. The comprehensive literature review by Chen *et al.* (2008) also uncovered previous studies that found calcium, sodium, magnesium and potassium ions to alleviate ammonia toxicity. However, the mechanism responsible for the improvement was not mentioned and remained unknown.

In this study, free ammonia concentrations in the pH-unadjusted wastewater treated with natural clinoptilolite zeolite were higher than the pH-unadjusted control at the end of the digestion while the ammonium-nitrogen concentrations remained unchanged in the zeolite-treated pH-reduced wastewater. While these observations suggested that the zeolite doses applied were inadequate in reducing the high concentrations of ammonium-nitrogen and free ammonia to levels below the ammonia inhibition threshold (section 2.5.4 in Chapter 2), the doses demonstrated stimulatory effect on methane production and VFA reduction. It is hypothesized that a combination of microbial immobilisation and stimulation by unknown exchanged cations released from the zeolite during the 10 days batch digestion were likely

factors that had contributed to the beneficial effects observed at these zeolite doses. The hypothesis of microbial immobilisation is supported by Fernández *et al.* (2007) who used scanning electron microscopy to provide strong evidence of natural zeolite serving as anaerobic microorganisms immobiliser in anaerobic fluidised reactors treating vinasses.

The Castle Mountain natural zeolite used in this experiment had been shown to have an ammonia-nitrogen uptake concentration of 4.5 mg-N/g zeolite on DiCOM recyclate, reducing its initial ammonia-nitrogen concentration of around 2.2 g-N/L to 1.3 g-N/L with 200 g/L zeolite in the thermophilic (55^{o}C) batch digestion of 80 minutes (Charles, unpublished). However, its effect on methane production was not investigated. Taking into consideration the results of this study and the findings of Milan *et al.* (2001) as well as Tada *et al.* (2005) on methane production inhibition at zeolite concentration above the optimum, it can be reasonably assumed that further enhancement of methane production and reduction of ammonium or ammonia concentrations might be possible at zeolite concentrations above the maximum 20 g/L applied in this study. However, intensive testing will need to be conducted to establish the optimum zeolite concentration that produces maximum ammonium and free ammonia removal but not inhibiting methane production at the same time. In addition, an in-depth study into the mechanism responsible for zeolite enhancement effect should also be carried out to get a better insight into its enhanced performance in wastewater treatment.

8.4.4. Effect of humic acid supplements on methane production from thermophilic batch anaerobic digestion of pH-unadjusted and pH-reduced piggery effluent

The results from this study showed that humic concentrations at 10, 15 and 20 g/L adversely impacted the methanogenic activity and resulted in reduced methane production (Figure 8.38 (a)). The observed increased viscosity with increasing humic acid concentration could in part have contributed to the retardation of nutrients transport to the microorganisms besides substrates competition by humics-reducing bacteria over methanogenic *archaea* (Cervantes *et al.*, 2008). Similar observation of methanogenesis inhibition of acetate by increased concentrations of anthraquinone-2,

6-disulfonate (AQDS), a model quinone analogue had been reported by Cervantes *et al.* (2000) who observed the redox potential to increase substantially (+130 mV) to level way above the low redox potential required for methane production (-200 to -400 mV).

The small stimulatory effect on methane production rate observed at the lower doses of 1 and 5 g/L (Figure 8.38 (b)) in conjunction with enhanced VFAs degradation particularly at 1 g/L (Figure 8.40) in the pH-reduced piggery wastewater clearly demonstrated that humic acid at the applied low concentrations served as electron acceptors in the anaerobic degradation of volatile organic acids as electron donors. Scott *et al*. (1998) using electron spin resonance (ESR) measurements provided direct evidence that the soluble quinones within humic substances are the redox active reducible organic radicals that function as the electron-accepting moieties.

A diversity of humics-reducing microorganisms ranging from ferric-reducing to sulfate-reducing, halorespiring, methanogenic, thermal and hyperthermophilic as well as fermentative microorganisms has been shown by several researchers to transfer electrons derived from the oxidation of organic compounds and/or hydrogen to humic substances. They include the ferric-reducing bacteria *Geobacter metallireducers* and *Shewanella alga* (Lovley *et al*., 1996; Scott *et al.*, 1998), *Geobacter sulfureducens, Geobacter bremensis* and *Geobacter pelophilus* (Straub and Schink, 2003); sulphate-reducing bacteria *Desulfovibrio* G11; halospiring bacteria *Desulfitobacteria* PCE1 and methanogenic *archaea Methanospirillum hungatei* JF1 (Cervantes *et al.*, 2002); thermophilic *archaea Methanococcus thermolithitrophucus and Methanobacterium thermoautotrophicum as well as* the hyperthermophilic a*rchaea Pyrodictium abyssi*, *Pyrococcus furiosus*, *Archaeoglous fulgidus, Thermococcus celer, Methanopyrus kanleri* (Lovley *et al*., 2000); mesophilic fermentative bacteria *Propionibacterium freudenreichii*, *Enterococcus cecorum* and *Lactococcus lactis* (Benz *et al*., 1998).

The observed enhanced VFA degradation particularly propionate which is considered to be the most difficult VFA to degrade indicated an improvement in the redox potentials or thermodynamic conditions of the treated piggery wastewaters by the low humic acid concentrations applied which allowed propionate to be degraded. It implied that the high hydrogen concentration or partial pressure which inhibited the

propionate from being degraded in the control was being scavenged by hydrogen-utilising microorganisms such as methanogens or sulphate-reducing bacteria (SRB) to a low level that allowed the propionate to be degraded. Humics-reducing bacteria also utilise hydrogen as electron donor. However, comparison of the thermodynamics of the microbial utilisation of hydrogen as electron donor in Table 8.18 suggests that H_2-utilising SRB, methanogens and homoacetogens would be thermodynamically more competitive than humics-reducing bacteria in scavenging the hydrogen substrate by using sulphate or carbon dioxide as electron acceptors.

Table 8.18. Thermodynamic comparison of various anaerobic microbial groups using hydrogen as electron donors

Reaction	ΔG^o (kJ/mol)
$4H_2 + 2CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$ (by homoacetogens)	-95.0
$4H_2 + 2CO_2 \rightarrow CH_4 + 2H_2O$ (by hydrogenotrophic methanogens)	-131.0
$4H_2 + SO_4^{2-} \rightarrow S^{2-} + 4H_2O$ (by sulphate-reducing bacteria)	-151.0
$H_2 + AQDS \rightarrow AH_2QDS$ (by humics-reducing bacteria)	-44.4

(Sources: Cervantes *et al.*, 2000; Brock *et al*., 1993, Thauer *et al.,* 1977)

As humic acid at 5 g/L greatly elevated the organic content of the piggery wastewater in terms of COD concentrations compared to the lower concentration of 1 g/L and the control, humic acid at 1 g/L was considered optimum in this study for methane production enhancement and reduction of the piggery wastewater organic matter. It is worth noting that while concentrations below 1 g/L humic acid were not tested in this study due to time constraints, it is conceivable that they might exert higher stimulatory effect on organics degradation and methane production. Quinone analogue, AQDS has been demonstrated to enhance ferric oxide reduction by acting as electron shuttle at concentration as low as 0.5µM (0.2 mg/L) at hyperthermophilic conditions (Lovley *et al*., 1999) while concentrations above 5mM (2 g/L) inhibited methanogenesis (Cervantes *et al.*, 2000). In view of the suppression of methanogenesis by humics-respiration (Cervantes *et al*., 2000, 2008), the effect of humic acid at concentrations below 1 g/L on anaerobic digestion of piggery wastewater warrants further study in order to establish the optimum concentration that would enhance organics degradation without inhibiting methanogenesis.

8.5. CONCLUSIONS

The findings from the pH experiment highlighted the importance of pH reduction in lowering the high inhibitory free ammonia concentration of the piggery wastewater thereby stimulating the growth of the anaerobic microbial consortia. Reduction of the piggery wastewater pH from 8.3 to 6.5 was demonstrated to be most effective in substantially enhancing microbial degradation of the elevated acetate and propionate, resulting in enhanced methane production rate. An unexpected, significant observation of the pH reduction approach was the decreased number of pathogenic *Clostridium perfringen* with increased methanogen population.

Supplementing 10% piggery biomass or DiCOM biomass to piggery wastewater without pH reduction was more effective in enhancing methane yield than at the higher volume of 19% which was subject to higher free ammonia and VFA inhibition. However, methane yield of the piggery biomass-supplemented wastewater without pH reduction was significantly lower than the control wastewater with pH reduction alone. Adding piggery biomass (10% and 19%) to the pH-reduced wastewater depressed the final methane yield due possibly to substrate limitation of the easily degradable organics as reflected by the lower final total VFA concentrations. On the other hand, adding DiCOM biomass (10% and 19%) stimulated the methane yields slightly due to its elevation of the wastewater total VFA concentration. Piggery biomass was considered a better choice as the DiCOM biomass contained significantly higher concentration of organic carbon compounds which greatly elevated the initial and final COD and VFA concentrations of the digested wastewater. On the basis of the elevated final TCOD concentration observed in the biomass-treated wastewaters, it was considered that pH reduction of the wastewater without biomass addition was a better option.

With zeolite treatment, 10 to 20 g/L zeolites stimulated methane production of the piggery wastewaters with and without pH reduction, with 20 g/L producing the highest enhancement effect. Reduction of the wastewater pH alone was found to be highly effective in reducing the ammonia concentration and also increasing the methane production. Zeolite-treated wastewater without pH reduction on the other hand, required up to 20 g/L zeolite to achieve comparable enhancement in methane production as the control pH-reduced wastewater. The enhancement effect of zeolite

was greater on the pH-unadjusted piggery wastewater than on the pH-reduced wastewater. A combination of microbial immobilisation and stimulation by unknown exchanged cations released from the zeolites were postulated to have contributed to the observed methane enhancement effects at the zeolite doses applied.

Humic acid concentrations at 10 to 20 g/L were strongly inhibitory to methane production from the piggery wastewaters with and without pH reduction. A low dosage of 1 g/L was found to be optimum for methane enhancement of pH-reduced wastewater and minimal elevation of the TCOD concentration.

Based on the outcomes of methane enhancement and the digested effluent quality, reduction of the initial pH of thermophilic piggery wastewater from pH 8.1 to 6.5 and zeolite treatment with or without pH reduction to 6.5 are effective strategies for enhancing methane production without elevating the final COD concentration of the treated piggery wastewater. However, pH reduction to 6.5 poses practical challenges from wasterwater foaming which needs to be taken into consideration when deciding which option to adopt.

CHAPTER 9
OVERALL CONCLUSIONS, RECOMMENDATIONS AND SCOPE FOR FUTURE STUDY

9.1. OVERALL CONCLUSIONS

The different reactor performance outcomes from thermophilic (55^{o}C) and mesophilic (37^{o}C) first-stage CSTR anaerobic treatment of low organic strength piggery wastewater and synthetic complex organic wastewater (4 g-TCOD/L) at 2-day HRT highlighted the important role wastewater characteristics play in the organics conversion efficiency of the anaerobic acidogenic reactors.

The vast differences in the physico-chemical and microbiological characteristics of the two types of wastewater were the key factors responsible for the first-stage thermophilic and mesophilic CSTR reactors producing different fermentation products and contrasting organics conversion efficiency. Unlike the synthetic complex wastewater that was prepared fresh with ground pig food pellets in deionised water, real piggery wastewater was comprised of a mixture of pigs' excrements (high ammonia-containing urea and faeces) plus rumen microflora, food droppings and wash water which had undergone further digestion in the ambient wastewater collection sump. As a consequence, the piggery wastewater contained very high initial levels of alkalinity, ammonium-nitrogen, soluble COD and volatile fatty acids (VFA) in conjunction with high levels of anaerobic microorganisms. The high alkalinity provided the piggery wastewater strong buffering capacity against VFA souring. On the other hand, the synthetic complex wastewater contained low initial concentrations of soluble organic matter (SCOD), negligible ammonium-nitrogen and lacked alkalinity, VFA and anaerobic microorganisms.

While mesophilic first-phase CSTR treatment of the synthetic complex wastewater hydrolysed and acidified more organic matter to VFAs than its thermophilic counterpart, the reverse was observed when raw piggery wastewater was treated in anaerobic first-phase reactors. Thermophilic treatment of the synthetic wastewater produced small amount of hydrogen and no detectable methane in the biogas which

indicated complete inhibition of methanogenesis. In contrast, both thermophilic and mesophilic treatment of the piggery wastewater produced methane which indicated that complete separation of acid-phase and methane-phase could not be achieved at 2-day HRT. The results from these experiments convincingly demonstrated that while synthetic wastewater has been and is extensively used in scientific studies to provide useful fundamental information, the highly complex chemical make-up of the real wastewater with its associated dynamic microbial interactions are characteristics which are extremely difficult to simulate with the synthetic wastewater. These key differences in the piggery and synthetic wastewater characteristics explain why the outcomes of the thermophilic and mesophilic first-phase acidogenic reactors treating the synthetic complex wastewater could not be replicated with the real piggery wastewater.

An attempt to turn the thermophilic anaerobic treatment of the piggery wastewater sour through increased feed concentration or organic loading rate from 4 g-TCOD/L (low-strength at OLR of 2 g-TCOD/L/d) to 7 g-TCOD/l (mid-strength at OLR of 3.5 g-TCOD/L/d) and maximum 13 g-TCOD/L (high-strength at OLR of 6.5 g-TCOD/L/d) was unsuccessful as more ammonia-nitrogen and bicarbonate were released from fermentation of the high levels of urea and protein components in the piggery wastewater which further increased the effluent alkalinity and pH. As a result, complete inhibition of the syntrophic consortia of VFA-consuming acetogenic bacteria and methanogens could not be achieved at the short HRT of 2-day as shown by the VFA reduction and concurrent methane production.

However, reducing the pH of high-strength piggery wastewater to pH 5.5 with concentrated hydrochloric acid proved to be effective in suppressing the activities of syntrophic consortia of acetogenic and methanogenic microbial populations and simultaneously stimulating the acidogenic bacteria. This was reflected by the lack of methane in the biogas and increased VFA concentrations in the reactor effluents. While this pH reduction strategy was effective in increasing the acidified soluble organic matter in the effluent and preserving the VFAs for methane production in the second-phase methanogenic reactor, the practical constraints posed by foaming-related spillages and the necessity to readjust the acidic effluent pH from 5.7 to

around neutral before it could be used as methanogenic substrate makes this approach unattractive to implement.

With more than 60% of the organics in the raw piggery wastewater as received already existed in soluble form and being completely acidified, the observation highlighted that the piggery wastewater collection sump at SARDI is essentially serving as an ambient pre-digestion or first-stage acidogenic reactor where hydrolysis of the particulate organic matter and acidification of the soluble organic matter to VFAs occur naturally.

Extending the operating HRT from 2-day to higher HRTs of 10- and 15-day for thermophilic treatment of undiluted piggery wastewater (11 g-TCOD/L) resulted in higher VFA removal and specific methane yield. However, no improvements were observed in the hydrolysis of the particulate organics and oxidation of propionate which remained at around 30% and 20% respectively of the total organic matter. The observed negative correlation between propionate degradation and free ammonia concentrations suggested that the syntrophic propionate-oxidising acetogens and hydrogenotrophic microorganisms were inhibited by the high free ammonia concentration released from the degradation of urea and proteinaceous organic compounds in the piggery wastewater. Particulate organics (29%), propionate (19%) and other non-VFA soluble organics (17%) formed the three largest groups of undegraded organic matter in the thermophilic digested piggery effluent at 15-day HRT.

In contrast, mesophilic anaerobic CSTR treatment of the undiluted piggery wastewater at 37^{o}C and 15-day HRT had low effluent free ammonia and total VFAs concentrations particularly propionate although the specific methane yield was significantly less than its thermophilic (55^{o}C) counterpart. The low levels of toxic free ammonia and VFAs in conjunction with the greater diversity and complexity of anaerobic microorganisms at mesophilic temperature were likely factors that have contributed to the improved thermodynamic conditions which allowed propionate oxidation to occur. Although the chemical quality of mesophilic effluent was superior to that of thermophilic effluent in terms of lower levels of soluble COD and VFA, mesophilic digestion is universally known for its ineffectiveness in pathogens destruction. Particulate organics (46%) and other non-VFA soluble organics (16%)

formed the two largest groups of undegraded organic matter in the mesophilic digested piggery effluent.

Thermophilic anaerobic batch vial experiments to study the effects of pH reduction, chemicals (zeolite and humic acid) and biological additions (piggery biomass and DiCOM municipal solid waste biomass) in enhancing organic carbon conversion to methane found pH reduction to 6.5 and treatment with 10 to 20 g/L natural zeolite with or without pH reduction were effective strategies to enhance methane production from the thermophilic-digested piggery effluent without elevating its final effluent COD concentration unlike biomass and humic acid. However, wastewater foaming poses a practical challenge for the pH reduction strategy.

9.2. RECOMMENDATIONS

Based on the findings from this research study on anaerobic digestion of synthetic complex wastewater and raw piggery wastewater, the following recommendations are made:

For laboratory-scale reactor results to be relevant, meaningful and realistic to a particular field application, it is imperative that the wastewater used in the study must come from the same source. The different reactor performance outcomes of the synthetic complex wastewater and raw piggery wastewater highlighted the important role wastewater characteristics play in determining the organics conversion efficiency of the anaerobic reactor and the type of acidified products formed.

Based on the observations of the high initial hydrolysis and acidification of organic matter in the raw piggery wastewater and the small increase in net hydrolysis following thermophilic treatment in the first-stage anaerobic CSTRs, a purpose-built first-stage anaerobic acidogenic reactor may not be necessary as the existing piggery wastewater holding sump on-site is already serving as an ambient pre-acidification system.

As CSTR is the common reactor type used for anaerobic treatment of livestock wastes and slurries, it is recommended that research study be carried out on ways to prevent propionate accumulation and reduce ammonia toxicity in the thermophilic

single-stage CSTR anaerobic reactor. Upgrading the CSTR configuration by incorporating microbial immobilisation materials such as inert carriers, eg shredded rubber tyre (Budiastuti, 2004) or natural zeolite (Montalvo *et al.*, 2006) and with minimal or intermittent stirring (Ward *et al.*, 2008; de Bok *et al.*, 2004; Kim *et al.*, 2002; Hansen *et al.*, 1998) is one way to facilitate close microbial proximity for effective interspecies electron or hydrogen transfer process to enhance propionate degradation. Zeolite at high enough concentration also serves as ion exchanger to reduce ammonia-nitrogen (ammonium and free ammonia) concentration. Control of the effluent pH to 6.5 is also one effective method to reduce the toxic free ammonia concentration as demonstrated in section 8.2.1 of this thesis, although one needs to be mindful about the foaming-related problems.

If zeolite is to be considered for ammonia control in piggery wastewater treatment, it is recommended that prior investigation is carried out to establish the optimum zeolite dose that would provide not only maximum removal of ammonia-nitrogen but more importantly maximum enhancement in methane production. Previous studies have found that zeolite concentrations above the optimum for enhancement of methane production resulted in inhibition of methanogenesis process (Milan *et al.*, 2001; Tada *et al.*, 2005). Fundamental study into the mechanism responsible for the process improvement should also be undertaken.

The electron-shuttling effect of humic acid at concentrations below 1 g/L on anaerobic digestion of piggery wastewater warrants further study to establish the optimum concentration that would enhance both organics degradation and methane production. Previous studies have demonstrated that the model quinone analogue, AQDS at concentrations above 5mM (2 g/L) inhibited methanogenesis (Cervantes *et al.*, 2000).

More research is required on the molecular FISH and real-time PCR methods to identify the cause of the substantially higher methanogen results produced by FISH over PCR results. The adequacy of sample fixation using paraformaldehyde on FISH probe permeability through cell walls of Gram-positive bacteria in particular, needs to be further investigated and compared with sample fixation using ethanol. This is to minimise the underestimation of cell numbers arising from probe permeability problem particularly with the cell walls of Gram-positive *Bacteria* (Bottari *et al.,*

2006; Kurisu *et al*., 2002; Amann *et al*., 1997). The use of standardised digital image processing and analysis software for automated quantification of fluorescent cells should also be looked into to replace the tedious and time-consuming manual counting which is prone to subjectivity.

While characterisation of the phylogenetic bacterial groups by FISH and T-RFLP profiling methods have provided some useful insights into microbial community structure in the piggery wastewater, targeting particular species responsible for specific functions eg, syntrophic propionate-oxidising bacteria and hydrogenotrophic methanogens or sulphate-reducing bacteria for propionate reduction/accumulation or particular species of pathogenic bacteria, eg, *Clostridium spp*. and/or *Campylobacter* as indicators of the level of hygiene achieved in relation to process (HRT, temperature) or environmental changes (eg, pH adjustment, biomass addition, zeolite treatment) would have added more practical value to the community structure data collected and the reactors' digestion performance assessment.

9.3. SCOPE FOR FUTURE STUDY

With 29% of the particulate organics still remained undissolved in the thermophilic digested piggery effluents at 15-day HRT, research is warranted in the area of pre-treatment methods (physical and/or chemical) to enhance the solubilisation of particulate organic matter for VFA and methane production. Alkaline chemical pre-treatment (Angelidaki and Ahring, 2000; Lin *et al*., 1997) in combination with physical methods such as thermal, ultrasonic, ball milling and maceration (Lee *et al*., 2009; Liu *et al*., 2009; Delgenes *et al*., 2000; Hartmann *et al*., 2000; Angelidaki and Ahring, 2000; Chiu *et al*., 1997) have been successfully tested on municipal sludge and animal manures to enhance the solubilisation of particulate organic materials. As the piggery wastewater also contained high level of ammonia-nitrogen which inhibits the anaerobic microorganisms, particularly at themophilic conditions, pre-treatment method should also look into means to reduce the ammonia-nitrogen level. Some methods that have been demonstrated to be effective in reducing ammonia-nitrogen are the use of zeolite to adsorb the ammonium ions (Cintoli *et al*., 1995) and precipitation of ammonium ions as struvite ($MgNH_4PO_4.6H_2O$), a slow-release non-burning fertiliser (Uludag-Demirer *et al*., 2008; Kim *et al*., 2004c).

Alternatively, research into post-treatment methods to reduce the high ammonia-nitrogen level and to improve the conversion of the insoluble particulate organics and soluble organics, particularly propionate in the thermophilic digested effluent is recommended to recover the residual methane and to improve the treated effluent quality. Some examples are post-anaerobic batch treatment of the thermophilic-digested effluent at the same process temperature as the main single-stage anaerobic reactor to enhance hydrolysis of the particulate organic matter (Angelidaki *et al*., 2005; 2006); use of zeolite as ion exchanger to adsorb the ammonia and ammonium ions (Sánchez *et al*, 1995; Milan *et al*., 1997); precipitation of ammonium ions as struvite (Uludag-Demirer *et al*., 2005; Nelson *et al.*, 2003; Miles and Ellis, 2001) or pH reduction to 6.5 as demonstrated in section 8.2 of this thesis. Reduction of the ammonia-nitrogen level in the digested piggery effluent will also be beneficial for the downstream integrated aquaculture system as the three major organisms - algae, zooplankton and fish can only tolerate total ammonia up to certain levels (Internal Quarterly Report for EBCRC Technical Advisory Committee: August-October 2005).

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APPENDICES

Appendix 1. Phylogenetic-related genera in Gram-negative Proteobacteria group (purple bacteria)

Group	Major genera with selected characteristics
Alpha-Proteobacteria (non-sulfur purple bacteria)	*Brucella*, a pathogen for animals, including humans *Caulobacter*, prosthecate bacterium with holdfast that grows in aquatic environments *Hyphomicrobium*, prosthecate bacterium that may divide by budding *Nitrobacter*, aerobic oxidisers of nitrite to nitrate (nitrifying bacteria) *Rhizobium,* fixes nitrogen when grown in leguminous root nodules **Rhodospirillum*, photosynthetic bacteria that have purple photo-pigments and do not use sulphur as an electron donor (purple non-sulfur); **Rhodopseudomonas, *Rhodobacter, *Rhodomicrobium, *Rhodopila* *Rickettsia*, obligate intracellular parasites that may have invertebrate vectors *Acetobacter, Agrobacterium, Aquaspirillum, Beijerinckia, Paracoccus, Pseudomonas* (some species)
Beta-Proteobacteria (non-sulfur purple bacteria)	*Bordatella,* a pathogen for animals, especially mammals including humans *Leptothrix,* bacteria with sheaths containing iron oxide and manganese oxide *Neisseria,* saprophytic or pathogenic bacteria that grow in mucus membranes *Nitrosomonas,* an aerobic autotroph that oxidises ammonia to nitrite *Sphaerotilus,* a sheath-forming bacterium that generally is devoid of metal oxides *Thiobacillus,* autotrophs that oxidise sulphur and thiosulfate to sulphuric acid **Rhodocyclus, *Rhodoferax, *Rhodovivax, Spirillum, Alcaligenes, Pseudomonas*
Gamma-Proteobacteria (sulphur purple bacteria)	*Azotobacter*, aerobic soil bacterium that fixes nitrogen under non-symbiotic conditions **Chromatium*, photosynthetic bacterium that have purple photo-pigments and can use sulphur as an electron donor (purple sulphur bacteria) *Escherichia, Enterobacter, Klebsiella, Proteus, Yersinia, Shigella, Salmonella* and *Serratia*, enteric bacteria f humans and somes strains pathogenic *Haemophilus*, many strains pathogenic for mammals and require blood factors for growth *Methylococcus*, aerobic oxidisers of methane, methanol, formaldehyde (methylotrophs) *Pseudomonas*, broad carbon utilisation and some strains produce opportunistic infections in animals; *Vibrio, Legionella, *Thiospirillum* and *other purple sulphur bacteria *Beggiatoa*, sulphide-oxidising bacteria which store sulphur intracellularly; *Leucothrix*
Delta-Proteobacteria	*Bdellovibrio,* prokaryotic predator that grows in the periplasmic space of Gram-negative bacteria *Desulfovibrio vulgaris, Desulforhabdus amnignus, Desulfuromonas* and other sulphate-reducing bacteria - uses sulphate and sulphur as electron acceptors with generation of H_2S *Myxococcus,* aerobic bacterium that may form fruiting bodies containing resistive cells *Synthrophobacter spp, Smithella propionica, Syntrophus gentianaeas.* *Pelobacter acetylenicus* – syntrophic propionate-oxidising bacteria
Epsilon-Proteobacteria	*Campylobacter* - many species pathogenic to humans and mammals *Helicobacteria* - pathogenic to humans and mammals

Continue

Group	Major genera with selected characteristics
Epsilon-Proteobacteria	*Thiovulum, Wolinella*

Photosynthetic
(Sources: Barton, 2005; De Bok *et al*., 2004; Brock *et al*., 1994)

Appendix 2. Phylogenetically-related genera in Gram-positive G+C bacteria group

Group	Major genera
Low GC bacteria	*Clostridium* and relatives: *Clostridium, Bacillus, Desulfotomaculum thermobenzoicum*, Thermoactinomyces, Sporosarcina, Acetobacterium, Streptococcus, Enterococcus, Peptococcus, Lactobacillus, Lactococcus, Staphylococcus, Ruminococcus, Planococcus, Mycoplasma, Acholeplasma, Spiroplasma, Pelotomaculum thermopropionicum*, Thermoanaerobacter brockii*, Syntrophospora bryantii*, S*train *Syn7*,* Strain HP*1.1**
High GC bacteria	*Actinomycetes*: *Actinomyces, Bifidobacterium, Propionibacterium, Streptomyces, Nocardia, Actinoplanes, Arthrobacter, Corynebacterium, Mycobacterium, Micromonaspora, Frankia, Cellulomonas, Brevibacterium*
Phototrophic subdivision	*Heliobacterium, Heliobacillus*

* Syntrophic propionate-oxidising (degrading) bacteria

(Sources: De Bok *et al*., 2004; Brock *et al*., 1994)

Appendix 3.1. Data comparison of piggery wastewaters (from the same batch) fed to lab-scale thermophilic reactor at 2-, 10- and 15-d with piggery wastewaters (from different batches) to pilot-scale thermophilic reactor at 7-day HRT (Trial 1)

Piggery feedwater to	Lab-scale	Pilot-scale*	Pilot-scale*	Pilot-scale*	Lab-scale	Lab-scale
HRT (day)	2	7	7	7	10	15
Parameters	Mean (± std dev)	Minimum	Maximum	Mean	Mean (± std dev)	Mean (± std dev)
pH	7.3 (0.1)	7.5	7.9	7.8	7.7 (0.1)	7.7 (0.1)
Total alkalinity (mg $CaCO_3$/L)	5595 (488)	na	na	na	4938 (159)	4550 (0)
Total COD (g/L)	12.9 (0.8)	4.7	36.2	18.2	10.6 (0.5)	11.0 (0.6)
Soluble COD (g/L)	7.6 (0.7)	4.3	25.2	12.4	6.1 (0.2)	7.3 (0.4)
Total VFA (g/L as COD)	8.1 (0.3)	0.1	1.8	1.2	5.8 (0.1)	5.6 (0.3)
Total Kjeldahl nitrogen (mg/L)	na	2500	4600	3300	na	na
Ammonium-nitrogen (mg/L)	1800 (0)	900	1800	1300	1941 (66)	1977 (21)
Free ammonia (mg/L)	4 (1)	na	na	na	10 (2)	10 (1)
Total phosphorus (mg/L)	na	111	158	132	na	na
Soluble phosphorus (mg/L)	112 (26)	42	97	77	na	na
Sulphate (mg SO_4^{2-}/L)	1200	na	na	na	na	na
Total solids (g/L)	na	3.8	16.1	8.8	8.0 (0.1)	8.1 (0.2)
Volatile solids (g/L)	na	1.8	12.1	5.9	5.1 (0.1)	5.0 (0.2)
Total suspended solids (g/L)	3.5 (0.8)	na	na	na	3.4 (0.2)	3.4 (0.1)
Total volatile solids (g/L)	3.2 (0.7)	na	na	na	3.4 (0.5)	3.0 (0.1)
Individual C2 to C6-VFA (mg/L as COD)	See Table 7.4	na	na	na	See Table 7.4	See Table 7.4

* Internal Quarterly Report for the EBCRC Technical Advisory Committee (August-October 2005), SARDI

na = not available

Appendix 3.2. Data comparison of lab-scale thermophilic reactor effluents at 2-, 10- and 15-d with pilot-scale thermophilic reactor effluents at 7-day HRT (Trial 1)

Reactor effluent	Lab-scale	Pilot-scale*	Pilot-scale*	Pilot-scale*	Lab-scale	Lab-scale
HRT (day)	2	7	7	7	10	15
Parameters	Mean (± std dev)	Minimum	Maximum	Mean	Mean (± std dev)	Mean (± std dev)
pH	8.1 (0.1)	na	na	na	8.3 (0.1)	8.4 (0.1)
Total alkalinity (mg $CaCO_3$/L)	6500 (165)	na	na	na	5533 (142)	5894 (8)
Total COD (g/L)	10.4 (0.8)	.3.8	8.6	6.7	7.9 (0.6)	7.1 (0.5)
Soluble COD (g/L)	7.1 (0.4)	1.3	7.4	5.0	4.9 (0.2)	4.9 (0.4)
Total VFA (g/L as COD)	6.0 (0.3)	0.26	1.5	0.76	3.5 (0.1)	3.0 (0.3)
Total Kjeldahl nitrogen (mg/L)	na	2400	3600	2900	na	na
Ammonium-nitrogen (mg/L)	2150 (191)	560	1700	1200	2111 (56)	2083 (42)
Free ammonia (mg/L)	788 (85)	na	na	na	920 (127)	1026 (74)
Total phosphorus (mg/L)	na	60	133	93	na	na
Soluble phosphorus (mg/L)	34 (5)	28	100	62	na	na
Sulphate (mg SO_4^{2-}/L)	590	na	na	na	na	na
Total solids (g/L)	na	4.8	8.9	6.1	7.2 (0.2)	7.5 (0.2)
Volatile solids (g/L)	na	1.9	6.0	3.5	4.1 (0.2)	4.3 (0.2)
Total suspended solids (g/L)	2.7 (0.5)	na	na	na	2.6 (0.2)	2.7 (0.2)
Total volatile solids (g/L)	2.3 (0.6)	na	na	na	1.9 (0.2)	1.9 (0.2)
Individual C2 to C6-VFA (mg/L as COD)	See Table 7.4	na	na	na	See Table 7.4	See Table 7.4
% methane in biogas	72 (1)	42	60	49	73 (1)	71 (2)

* Internal Quarterly Report for the EBCRC Technical Advisory Committee (August-October 2005), SARDI

na = not available

Appendix 4.1. Data comparison of piggery wastewaters (from the same batch) to lab-scale thermophilic reactor at 2-, 10- and 15-d with piggery wastewaters (from different batches) to pilot-scale thermophilic reactor at 4-day HRT (Trial 1)

Piggery feedwater to	Lab-scale	Pilot-scale*	Pilot-scale*	Pilot-scale*	Lab-scale	Lab-scale
HRT (day)	2	4	4	4	10	15
Parameters	Mean (± std dev)	Minimum	Maximum	Mean	Mean (± std dev)	Mean (± std dev)
pH	7.3 (0.1)	7.5	7.8	7.7	7.7 (0.1)	7.7 (0.1)
Total alkalinity (mg $CaCO_3$/L)	5595 (488)	na	na	na	4938 (159)	4550 (0)
Total COD (g/L)	12.9 (0.8)	7.0	33.2	20.4	10.6 (0.5)	11.0 (0.6)
Soluble COD (g/L)	7.6 (0.7)	6.1	11.5	8.3	6.1 (0.2)	7.3 (0.4)
Total VFA (g/L as COD)	8.1 (0.3)	0.9	6.1	2.7	5.8 (0.1)	5.6 (0.3)
Total Kjeldahl nitrogen (mg/L)	na	2900	4900	4700	na	na
Ammonium-nitrogen (mg/L)	1800 (0)	900	1800	1400	1941 (66)	1977 (21)
Free ammonia (mg/L)	4 (1)	na	na	na	10 (2)	10 (1)
Total phosphorus (mg/L)	na	127	160	144	na	na
Soluble phosphorus (mg/L)	112 (26)	72	126	98	na	na
Sulphate (mg SO_4^{2-}/L)	1200	na	na	na	na	na
Total solids (g/L)	na	7.5	26.2	15.5	8.0 (0.1)	8.1 (0.2)
Volatile solids (g/L)	na	4.7	20.6	11.4	5.1 (0.1)	5.0 (0.2)
Total suspended solids (g/L)	3.5 (0.8)	na	na	na	3.4 (0.2)	3.4 (0.1)
Total volatile solids (g/L)	3.2 (0.7)	na	na	na	3.4 (0.5)	3.0 (0.1)
Individual C2 to C6-VFA (mg/L as COD)	See Table 7.4	na	na	na	See Table 7.4	See Table 7.4

* Internal Quarterly Report for the EBCRC Technical Advisory Committee (August-October 2005), SARDI

na = not available

Appendix 4.2. Data comparison of lab-scale thermophilic reactor effluents at 2-, 10- and 15-d with pilot-scale thermophilic reactor effluents at 4-day HRT (Trial 2)

Reactor effluent	Lab-scale	Pilot-scale*	Pilot-scale*	Pilot-scale*	Lab-scale	Lab-scale
HRT (day)	2	4	4	4	10	15
Parameters	Mean (± std dev)	Minimum	Maximum	Mean	Mean (± std dev)	Mean (± std dev)
pH	8.1 (0.1)	na	na	na	8.3 (0.1)	8.4 (0.1)
Total alkalinity (mg $CaCO_3$/L)	6500 (165)	na	na	na	5533 (142)	5894 (8)
Total COD (g/L)	10.4 (0.8)	.5.0	17.9	10.0	7.9 (0.6)	7.1 (0.5)
Soluble COD (g/L)	7.1 (0.4)	2.0	11.3	6.8	4.9 (0.2)	4.9 (0.4)
Total VFA (g/L as COD)	6.0 (0.3)	0.24	2.3	1.1	3.5 (0.1)	3.0 (0.3)
Total Kjeldahl nitrogen (mg/L)	na	2400	4000	2900	na	na
Ammonium-nitrogen (mg/L)	2150 (191)	580	2000	1300	2111 (56)	2083 (42)
Free ammonia (mg/L)	788 (85)	na	na	na	920 (127)	1026 (74)
Total phosphorus (mg/L)	na	113	129	121	na	na
Soluble phosphorus (mg/L)	34 (5)	75	107	87	na	na
Total solids (g/L)	na	4.5	10.6	7.6	7.2 (0.2)	7.5 (0.2)
Volatile solids (g/L)	na	2.3	7.3	4.8	4.1 (0.2)	4.3 (0.2)
Total suspended solids (g/L)	2.7 (0.5)	na	na	na	2.6 (0.2)	2.7 (0.2)
Total volatile solids (g/L)	2.3 (0.6)	na	na	na	1.9 (0.2)	1.9 (0.2)
Individual C2 to C6-VFA (mg/L as COD)	See Table 7.4	na	na	na	See Table 7.4	See Table 7.4
% methane in biogas	72 (1)	48	58	52	73 (1)	71 (2)

* Internal Quarterly Report for the EBCRC Technical Advisory Committee (August-October 2005), SARDI

na = not available

Appendix 5. Phylogenetically-related genera in the T-RFLP fragments of bacterial groups

Bacterial group	*Alu* Fragment	Genera
A	270	*Cytophaga*, a group of facultatively anaerobic lower myxobacteria, belonging to the CFB group and Bacteriodes phylum. A strictly anaerobic fermentative bacterium *Anaerophaga thermohalophila* which ferments hexoses and pentoses to acetate, propionate and succinate.
B	387	Several groups possibly including some *Cytophaga* species, *Prevotella* which can be strictly anaerobic, ferments pentoses; *Porphyromona*, a strict anaerobe ferments sugars to butyric acid, iso-valeric acid, succinic acid, phenylacetic acid.
C	576	Several groups, possibly including *Desulfovibrio*, a strict anaerobic sulphate-reducing bacterium; *Desulfosporosinus*; *Bifidobacterium*, eg, *Psychroaerophilum* fermentative, facultative anaerobe isolated from a pig caecum; *Treponema*, eg *succinifacien*s, an anaerobic spirochaete previously isolated from swine intestines; or an anaerobic bacillus.
D	159	An anaerobic *Planctomycete*, capable of anaerobic ammonia oxidation.
E	249	Uncultured group of non-sulfur bacteria, *Chloroflexi* often abundant in wastewater treatment processes.
F	307	Other species of *Planctomycetes*
G	282	*Meiothermus silvanus/Thermotoga/Acinetobacter/ thermodesulfobacterium.*
H	402	*Thermotoga spp.* extreme thermophiles, *thermodesulfobacterium* is a Group II sulphate-reducing bacteria as it can utilise acetate and other fatty acids, oxidising them completely.
I	420	*Prevotella spp./Planctomycetes*
K	171	Cyanobacteria (*Phormidium*) which can grow anaerobically at moderately thermophilic temperatures or *streptomycete*.
L	216	Large grouping which includes *streptomyces*, *Cellulomonas*,*Gordonia*, *Corynebacterium*, *Microbacterium*, *propionibacterium*.

(Courtesy of Skillman)

Appendix 6. Phylogenetically-related genera in the T-RFLP fragments of bacterial groups

Fragment size (bp)	Putative Identification
62	*Rhodothermus*, *Picrophilus*
87-9	Large grouping which includes the propionate producers *Viollenella* and *Megasphaera*
112	*Propionibacterium, Streptomyces*
137	*Desulfotomaculum*, *Pseudoalteromonas*, *Clostridium*
150-6	*Cytophago*, *Clostridium*
171	Cyanobacteria (*Phormidium*) or streptomycete
216	Large grouping which includes *streptomyces*, *Cellulomonas*,*Gordonia*, *Corynebacterium*, *Microbacterium*, *propionibacterium*
249	*Meiothermus ruber*, *Clostridium*, *Thiobacillus*
258	*Rhodococcus, Pseudomonas, Cytophago, Alteromonas*
269	*Cytophaga, Anaerophaga thermohalophila*
274	*Myxococcus, Capnocytophaga, Clostridium*
282	*Meiothermus Silvanus/Thermotoga/Acinetobacter/ thermodesulfobacterium*
307	*Planctomycetes*
321	*Salmonella, Pseudomonas*
359	*Calothrix, Meiothermus*
386	*Cytophaga* species*: Prevotella, Porphyromonas*
402-10	*Thermotoga* spp. extreme thermophiles*, Thermodesulfobacterium*
419	*Prevotella* spp*./Planctomycetes*
426	*Bacillus, Streptomyces*
485	*Cytophago, Bacteroides*
516	*Bifidobacterium, Actinobacillus, Desulfonatobacterium hydrogenovorans*
560	*Desulfovibrio, Desulfosporosinus, Bifidobacterium* eg. *Psychroaerophilum*

(Courtesy of Skillman)